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CANCER RESEARCH

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This Business of Cancer Research*†

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For many years our presidents have devoted their presidential addresses to the stimulating scientific attainments of their laboratories. I am going to deviate from that custom, and devote myself to the present state of publicly endowed research on cancer—its relation to the investigator himself, and his relations to the university and to the public. For some years the members of this society have been receiving supplementary financial support in unprecedented amounts from the public, primarily from government agencies and from the publicly supported American Cancer Society, as well as from private agencies such as the Childs Fund. Because of these funds, we have left behind our previous state of perpetual penury and have been able to multiply our work, to take young men into our laboratories, to build new laboratories, and to improve our equipment. Above everything else we can make long term plans for efficient progress in research and have been able to acquire confidence that young men will have good opportunities for doing constructive research in this field and, therefore, that it is a good field for them to enter and develop. This society has accepted these changes practically without official comment. But I think the members of this society should explore the changes which are occurring, should have opinions as to their significance and virtue. It is to this that I will dedicate this short address. I think our first duty and pleasure is to thank all of those who, in the last 6 years, have done such outstanding work in collecting and allocating the public money entrusted to them. We thank them with enthusiasm —their work has been done with generosity, with a universally impartial approach and a desire to get on with the problem. I know of no effort to organize help on a scientific problem which has had greater success, which has been done with greater wisdom, or which deserves greater praise. But it is time the investigator looked at his own life and work and evaluated their relation to these new blessings.

The generous amounts of new funds should be used with restraint, as we have been trained to do by our previous state of poverty. We should ask only for what we really need. We should ask for public funds as we ask for university funds, with a generous spirit towards others who also need expansion, who also have good ideas worthy of support. If we maintain our academic conscience, if we can only continue to be simple in our purposes and our work, then public support may continue. If we do not do these things, you can be sure that the critical eyes of investigating committees will disclose our errors, and we will lose caste. This is but just.

What has this increased affluence done to scientists? For one thing, it has changed the life of the head of the department. He finds that he has added administrative, financial, and personnel duties to the already full life of a scientist and teacher. He is involved in much travel and many committees. He is engulfed in writing requests for funds and then in justifying their expenditure. These diversifications of responsibility which spread his energies over many fields cause him to ponder the merits of the system. Probably his one desire is to work quietly in his laboratory and train his young men and women, and certainly that is his primary responsibility.

With all these increased demands on laboratory

* Presidential address delivered at the Forty-first Annual Meeting of the American Association for Cancer Research, Inc., Atlantic City, N.J., April 17, 1950.

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heads, however, too few new permanent posts for mature men have been created. There is little difficulty these days in obtaining fellowship support for promising young scientists. Before cancer research received public financial support, loyalty to younger men made the professor show them the difficulties in the field—the lack of public interest, the difficulty of university and hospital promotion for those working in diseases which come slowly and hence are solved slowly, and so the general precariousness of the life. This is disappearing now—I hope for good. He can speak with enthusiasm about a life spent in this work. Opportunities seem to be as good in this field as in any. Certainly the diseases of advancing years are the problems which this next generation must try to solve.

I have always thought that a professor's first responsibility is to his young associates. I find it hard to believe that the scientific problem is more important than the individual attacking it. They are both important, and so we must have a certain increasing number of respectable jobs for these young men to attain. The older teacher needs relief, the younger men need the opportunity.

The many fellowships which are available now temporarily solve the problems of support for young men and their laboratories. This support gives youth the opportunity which it has the right to demand, a chance to work and to compete in an interesting scientific field. One cannot, however, expect men to serve on a fellowship basis year after year. Cancer research demands continuity of effort which can only be maintained by having assurance of support for seasoned workers who can form the permanent nucleus of the program. Assured continuity is necessary to undertake many important problems, and prolonged grants are necessary to assure their completion. Only the better men can expect to stay indefinitely in university work. But serving as fellows for years delays the decisions as to who deserves to be retained. Fundamental science has never thrived on mass production, and it is to be expected that it is the rare, original individual who should continue to be cherished. A private university cannot be expected to give tenure of office unless assured funds for it are available, and institutions which acquire funds on a yearly basis are in poor position to offer the security of such senior posts. At present this is an impasse, a problem which must be solved.

What are some effects on the university? Certainly the availability of public funds for specific purposes presents problems as well as opportunities for an integrated university. An institution relying on a constant flow of funds for its permanent commitments now receives a large percentage of its research funds in the form of added annual grants. Many of these grants are voted by committees which are unrelated to the university. By this procedure, progress in the specific problems is undoubtedly furthered to an extent which is surprising to us older men who have been accustomed to the plodding methods of the prewar university laboratories. The budget of some individual department thrives if it has a specialized discipline which adapts to a specialized problem. But one must be careful not to disturb the balance between departments too much, a balance which is obviously important to well integrated medical education.

At the present time, a fair balance between annual grants and the newer institutional grant seems wise to me. This newer type of grant has many advantages. It returns the decision of allocation of funds to institutions which merit confidence. This makes possible a local supervision, a unification which eliminates undesirable duplication of effort, and offers fluidity in the use of funds to stimulate and originate work. It is obvious that institutions have to learn to use these institutional grants solely for integrated research, and not as easily acquired funds for the reduction of deficits.

We think we are finally handling this type of grant properly at Harvard through an organization which was reestablished because of the institutional grant. A university committee has been appointed with satellite panels representing different scientific disciplines and chosen on a university-wide basis. This committee advises the administration where funds can be most effectively used, particularly with a view to interdepartmental research. So far, this has been limited to venture research, so-called pilot experiments, but it might be wise to use such money for more prolonged collaborations.

Such committees tend to break down departmental barriers, even those between faculties, and they may indeed integrate and produce joint effort among men from different universities. The advantages of serving on the committee and its panels are great. We are planning dinner meetings for about twenty-five men from various local institutions to discuss informally various problems of growth. Nothing can be more desirable than the collaboration of heads of departments, men with different disciplines, who thus focus on a common problem. When biologists, chemists, and physicists discuss problems of growth with their medical school confreres, their different conceptions of why reactions occur prove very stimulating and mutually beneficial, even though they

usually make the problem look more complex. This type of integration is more apt to come about when a grant is given to an institution rather than to a department or to an individual in the institution. It is possible that the fundamental difference between the two types of grant lies in the use of the institutional grant for pilot experiments and the individual grant for established projects which are producing results.

Interdepartmental discussion puts research on a firmer basis. We learned during the last war that cooperation is apt to be profitable. We have learned that departmental barriers were really only budgetary, that collaboration with others interested in the same problems could broaden the horizons of our work and could make most interesting fundamental research. For example, our department has intimate and profitable contacts with the Departments of Anatomy and Biochemistry at the Harvard Medical School, with the Department of Organic Chemistry at Harvard University, and with the Physics and Biology Departments at the Massachusetts Institute of Technology. This is of course not unique.

I think this has come about partially because of the great complexity of the problem, and the increasing conviction that it is going to take many minds to solve it. But it has resulted in many pleasant social contacts and in broadening our scientific horizons.

The individual investigator, indeed the single department, may often work on fact-finding problems without regard to theory. I approve of this, for it pushes a problem forward. But discussions of such work with men in other fields almost inevitably turn to theories of action, and such theories lead to scientific explorations. There is merit in such a result, which reminds me of the remark of Sir William Bayliss:

> "Truth is more likely to come out of error, if this is clear and definite, than out of confusion, and my experience teaches me that it is better to hold a well-understood and intelligible opinion, even if it should turn out to be wrong, than to be content with a muddle-headed mixture of conflicting views, sometimes called impartiality, and often no better than no opinion at all."

Not only are research men working harmoniously and profitably together, but they have also found firm support and genuine interest from the public, due partly to stimulation by the daily press. Many members of the press have devoted themselves to helping raise the money we use in our research and, in this, their efforts are invaluable. There is no comparison between the present able science writer and the somewhat unreliable neophyte of 15 years ago. Many present writers for the daily papers know what they are writing about, are well educated in science, and know also what appeals to the public. One of the best ways to educate the public is by close collaboration between scientists and science reporters, but it has taken time to teach investigators that this is an important activity. I think we have nearly all come to realize it now, and want to thank the science writers for a difficult job conscientiously undertaken. Like research work on the problem, the technic of science reporting is growing up. There is great need that this movement should spread. The relationship has one difficulty—a universal pressure for a continuous flow of new and exciting news which is a demand that investigators cannot conscientiously meet. The most exciting news is apt not to be flamboyant, and most scientific discoveries about growth and cancer develop inconsistencies so that to be dogmatic is obviously dangerous. We should stop trying to thrill the public with repeated claims of new cures and discoveries and rather try to educate them about the known physiologic mechanisms of nature. How to do this in a quiet, dignified, reserved way is a problem of adult education which needs study. I am convinced that repeatedly interpreting small discoveries as exciting news will eventually reduce the public interest; and responsibility for this rests on investigators and societies, as well as on the press. Today, the society has established a committee to explore this problem of cancer publicity in the broadest sense and to report back to the Board of Directors. The cancer problem does not appear to be a simple one—at the moment it looks complicated. It takes months to years to do a single experiment, and even when a series is completed the answer is often hemmed in with doubts. The problem has proved to be a baffling one, and many experiments are unsuccessful. When a series of observations proves positive, it often has scientific interest only and may be of little interest to the public. Yet this type of discovery is apt to be the more important to science.

Present laboratory research can be divided roughly into two approaches to the problem. Chemotherapy is the urgent approach which always attracts public interest but which entails risk of being unsuccessful and misinterpreted. The other less dramatic approach is to devote efforts to learn more about the fundamental causes of cancer. This is essential to understand the problem, and even should a good new therapeutic procedure be discovered it will still be essential to ferret out the cause of the disease. If the cause is deep held, in chromosomes for instance, it will surely take a long time to solve. This we do not now know, but we must be preparing for a long search.

Our relations with the press ought to be predicated on this single constant premise, namely, that this is a very difficult problem which will take a long time to solve. Interesting discoveries will come more frequently, but, until a great fundamental discovery is made, our progress will be careful and plodding. It is wise for those involved in raising public funds to impress this on the public, and to emphasize the fact that this will be a slow hard fight, and one which both public and investigators must be prepared to wage for many years. False hopes which do not materialize may result in loss of public interest, and this would be a catastrophe to the large group of scientists who are dedicating their efforts to the cancer problem.

Would it not be wise to establish collaboration between scientists and publicists to embark on a quiet adult education program as well as to establish a nonpartisan clearing-house for informing the public? For instance, it might be well to write a series of articles on cell division throughout nature, on specialization found among cells and their subsequent interdependence, and on the effect of x-rays and other agents and what we know of their effects on life processes. Many such subjects could be covered. Indeed, the movement has started and such articles are beginning to appear in magazines and popular books. Many of our publicist friends who write for the daily papers say these do not reach people. This may be so at present, but there is obviously an increasing interest on the part of the public in scientific affairs, and it is well worth the trying. Unless we are very lucky, the present method of raising hopes which do not materialize will bore people before our job is done.

These various problems which confront us are not really fundamental, and they will be solved or corrected. The workers in this field are in an enviable position now, compared to their situation 10 years ago. At that time there was little interest, either public or academic, and opportunities for work were meager and discouraging. Now the work is really thriving.

This society reflects this growth. As one of our oldest members—a former president—has told me, the society was started to give men opportunity to talk of work they could not report elsewhere. In 1922, when the society had its fifteenth meeting, there were 138 members, nearly all of them surgeons and pathologists with but a passing interest in cancer. At the annual meeting that year there were nine papers, many of them reporting failure of experiments, and in 1932 there were eighteen papers. This year there are about 740 members and 142 papers on the program. Our journal, returned to the society in 1948, has ample worthy short papers submitted in increasing numbers. With the many continued generous subsidies given the journal and with its present able editorial leadership, its future appears assured. The purposes of this journal tie in with the two other excellent newer journals. All of this gives indication of our improvement in productivity and promise.

As scientists who now owe a report to the public, we can say that this problem demands the exploring of the finest metabolic characteristics of body cells, the very heart of biology. Even though brilliant discoveries may hurry us on our way, the real, thorough solution of this problem will be slow. There are always times when scientific problems can be attacked, and it appears that the time for productive work on this problem is ripe, that opportunities for good work are ample.

We are making progress with this baffling problem and should continue to do so with increasing speed. From the scientist's point of view, one cannot expect more.

Tumors in the Invertebrates: *A Review*

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INTRODUCTION

Tumors are the result of abnormal cell proliferation. Therefore, the study of tissue growth, normal and abnormal, constitutes the central problem of tumor research which thus becomes essentially a biological problem. When approached from this broader point of view, an analysis of tumorous growth should include representatives from all groups of living organisms. It has been recognized that the study of plant tumors yields significant results. Within the animal kingdom comparative pathology has concerned itself largely with neoplasms in various groups of vertebrates (74, 109, 112, 124), while invertebrates have been all but neglected. As a matter of fact, until fairly recently invertebrate tissues were often considered incapable of developing tumorous growths.

Teutschlaender (144) believed that tumors cannot occur in animals at a phylogenetic level lower than the fishes. Engel (25) elaborated extensively on the reasons why invertebrates are unable to develop cancer. Emphasizing anatomical differences between invertebrates and vertebrates he discussed chiefly three points. The first two concern differences in the nervous and vascular systems; the third deals with the embryonal theory of tumor growth. Believing that invertebrate cells, on account of their considerable regenerative power, are embryonic in character, Engel concluded that they cannot revert to the embryonic stage and produce tumors. In the light of modern biological concepts these views are obsolete, not only with regard to anatomical considerations, but also because they are based on inadequate material.

Actually, during the past 50 years a considerable literature on spontaneous and experimentally induced tumors in invertebrates has grown. In addition, numerous observations exist concerning various tissue reactions that may be more or less closely related to tumorous growth. These data are scattered and often not easily accessible. The only papers in which invertebrate tumors are reviewed to some extent are two in French (13, 148) and one in Russian (30).

A critical survey of the present status of the problem of invertebrate tumors encounters serious difficulties. For one thing, the data in the literature are often controversial, and many of the descriptions are inadequate or hard to evaluate. Pathologists specializing in tumor research are not, as a rule, familiar with invertebrate material. On the other hand, zoölogists, versed in the intricacies of invertebrate anatomy and taxonomy, are usually inexperienced in the diagnosis of tumor growth. Furthermore, the terminology developed almost exclusively for use in mammalian pathology should not be applied to invertebrate animals, until the analogies between vertebrate and invertebrate tissues are more thoroughly understood.

In spite of such difficulties two facts stand out as the result of this survey: invertebrate tissues are capable of tumorous change, and they offer an opportunity to approach the study of tumors in new ways.

TUMORS IN VARIOUS GROUPS OF INVERTEBRATES

The first question one may ask is: In which types of invertebrates have tumors or cellular reactions comparable to tumorous growth been observed? The information available refers to almost all the major invertebrate phyla. Tumors are said to occur in annelids, sipunculids, arthropods, molluscs, and ascidians. Even among the most primitive metazoans—dicyemids (91)—and among the protozoans (82) nuclear anomalies have been observed which are comparable to atypical mitoses as found in certain mammalian neoplasms. The best known group and the most interesting from the point of view of comparative oncology are the insects.

VARIOUS TISSUE REACTIONS AND TUMORLIKE GROWTHS

Great caution is indicated in an evaluation of the types of tumors occurring in invertebrate animals. For one thing, the difficulty of arriving at a

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satisfactory classification, which exists even in mammalian pathology, is multiplied in invertebrates. Furthermore, in a number of instances a variety of formations in invertebrates which cannot be considered as true tumors have been designated as such. Among these are cysts, often the result of what is called an "inflammatory process." It would be preferable to call such processes "injury reaction" or "repair hyperplasia," since according to Menkin (78) the term inflammation, which implies vascular response, should be restricted to vertebrates. A frequently observed situation is the following (e.g., 8, 11, 24, 31, 54, 56, 59, 60, 61, 64, 68, 69, 80, 83, 98): almost any disturbing factor (foreign body, parasite, tissue implant with or without bacterial contamination, degenerating organ, hereditary tissue anomaly, sterile agar or celloidin, or irritant, such as croton oil) will cause an accumulation of lymphocytes in the affected area. In many invertebrates certain blood cells may change from free-moving to sessile elements and vice versa under various conditions (27, 28, 73, 163). When they accumulate, often in impressive numbers in the vicinity of irritants, phagocytosis and encapsulation of the center of disturbance take place. In the cysts thus formed the cells making up the capsule may undergo changes, sometimes of a degenerative nature. Such tissue responses resulting in well defined "growths" may easily be mistaken for tumorous processes. In most cases they merely indicate an injury reaction, but it is not unlikely that under certain conditions such cysts may eventually give rise to tumor-like growths or real tumors.

Various abnormal growths, with or without ulceration, have been observed on the body wall of molluscs collected in nature or reared in the laboratory. The causes of these growths are unknown. In each case the histological picture showed the presence of an injury reaction with repair hyperplasia, characterized by densely packed nuclei of connective tissue cells, fibroblasts, and numerous migrating cells. Tissue reactions experimentally produced by burning and by strong acid were essentially the same as these natural growths (139, 141, 142).

Another type of cellular response in invertebrates which in itself should not be considered as tumorous growth manifests itself in the *hypertrophy* of cells due to the action of parasites. For example, in the intestinal epithelium of certain marine worms gregarines may cause up to a tenfold increase in cellular size accompanied by changes in the appearance of nuclei and cytoplasm (9, 10, 102, 136, 145).

Somewhat more significant in connection with tumorous changes is the effect of certain parasites on the so-called fat body of insects, a type of connective tissue which stores reserve materials and, in certain species at least, shows no sign of cellular proliferation after metamorphosis. Under the influence of the parasites these adipose cells have been observed to hypertrophy and to resume mitotic activity in the adult stage. Many of these mitotic figures are abnormal (67, 79, 106). The result is a *hyperplasia* of the fat body of these insects (16, 17, 18, 23, 99).

Another instance where caution in the use of terms is indicated is that of the insect "mycetomes" (structures caused by intracellular symbiotes). The practice of Mahdihassan (75, 76) of considering these biologically useful structures of bacterial origin as tumors is not in keeping with commonly accepted views (135, chapters 4 and 6; 137, chapter 5). Likewise, parasitic structures such as those described by McIntosh (77) in the caudal region of Sagitta (Chaetognatha) are not tumors in the strict sense of the word.

Many of these pathological formations are evidently not true tumors but may show certain resemblances to neoplastic growth. Thus, it is obvious that in many instances the decision as to whether or not a reported structure is to be classified as a neoplasm meets with considerable difficulty. A great deal of further observation is necessary before an opinion can be formulated. Just as certain tumors in vertebrates are considered to be on the borderline between hyperplasia and benign tumors, some of the formations reported here in the invertebrates might prove to be borderline cases.

Nevertheless, so far as can be judged from the available evidence, cases of true tumors, both benign and malignant, appear to occur among invertebrates. These cases are discussed in the following section, in which the term "tumor" will be freely used wherever the balance of evidence seems to suggest a true neoplastic growth. Also discussed in this section will be certain experimental work of relevant interest, including cases in which no neoplastic growth was obtained.

CAUSES OF INVERTEBRATE TUMORS AND TUMOR-LIKE STRUCTURES

Spontaneous occurrence.—A number of cases reported in the literature concern incidental observations of so-called spontaneous tumors. It is perhaps more accurate to classify them as tumors whose cause is unknown. Among the earliest observations is one on a lobster (Homarus) by McIntosh who "many years ago, described a tumour which originated in the wall of the grinding stom-

ach and pushed its way through the carapace behind the eyes. The tumour enlarged and finally resulted in the death of the lobster, which was a very large and old specimen."[1]

Kolosváry (65) found a tumor on the prosoma of an arachnid, *Phalangium opilio* L. The chitinous body wall produced an overlapping fold over the tumor; the latter was so large that it pushed the internal organs to one side. The author suggested that this tumor might have started during embryonic development. No histological study of the tumor was made.

Additional chance observations among arthropods concern insects. The earliest reference to insect "tumors" seems to be in a treatise on insect diseases by Kirby and Spence in 1826 (quoted from 137), Figure 1, taken from a monograph by Balazuc (4), shows a large tumor in the prothorax of a beetle (*Phytodecta variabilis*) of which no histological analysis is available. White (152) found a fibroma-like structure in the thorax of one honeybee among a large number of dissected specimens. The tumor appeared as a mulberry-like mass which displaced adjacent structures and seemed connected with the mesothoracic ganglion. However, the microscopic picture indicated that the tumor was derived from connective tissue rather than from ganglionic elements.

Likewise unknown was the cause of tumor-like structures which Örösi-Pál (92) described in the hindgut of old winter bees. They consisted of cysts formed by fusion of vacuolated giant cells whose nuclei did not seem different from those of normal hindgut epithelium.

A rather unique finding of a brain tumor in an ant (*Formica pratensis*) was described by Brun (6). The specimen, a worker, showed motor disturbances (continuous circular movements to the right) which suggested a cerebral lesion. On microscopic examination a compact tumor was found on the upper left side of the protocerebrum, taking the place of the corpora pedunculata. It consisted of very small, densely arranged cells, presumably proliferated glia elements. A differential diagnosis between tumor and brain abscess was, however, not possible on the basis of the available material.

A "unicellular tumor" was described by Palm (96) in one corpus allatum of a male nymph of *Gryllotalpa* (Orthoptera). Among the small cell elements of this endocrine organ giant cells occur which are derived from several smaller cells by cytoplasmic, and subsequently nuclear, fusion (20). In the case reported such a round, giant cell, which was clearly separated from the surrounding tissue, showed distinct signs of karyorrhexis. The nuclear membrane had disappeared, and numerous chromatin granules and nucleolar remnants were freely distributed in the cytoplasm. The cytoplasm appeared dense and homogeneous, exhibiting signs of hyaline degeneration. No visible influence of this condition on the health of the host could be detected. Although this pathological structure, whose diameter was 65 μ, took up about one-third of the total longitudinal diameter of the corpus allatum, it seems debatable whether or not it should be classified as a tumor.

Another case reported by Palm (97) concerns the pharyngeal glands of the bumble-bee, Bombus. One of the male specimens examined showed "tumor formation" in the gland of one side. Normally consisting of unicellular units equipped with separate canals leading to a common duct, the diseased gland appeared as a displaced, rather large, compact structure containing an abundance of connective tissue. There was no connection to the common duct; numerous aberrant small secretion canals ended blindly. The abnormally large glandular elements showed signs of hypersecretion and degeneration. Karyorrhexis occurred among the nuclei.

Among molluses, several tumors have been reported. Williams (155) and Collinge (14) found, among about one thousand examined specimens of the freshwater mussel (*Anodonta cygnaea*, var. zellensis), three with tumors. These growths were apparently derived from the tissues of the mantle and, in their microscopic structure, resembled adenomyomas. There was evidence that these tumors had seriously interfered with the physiology of the afflicted animals.

In the oyster (*Ostrea virginica*) G. M. Smith (127) observed, as had Ryder (118) before him, a benign tumor of mesenchymal character which had its origin in the pericardium. The pediculated structure, whose largest diameter was $1\frac{1}{4}$ inches, showed a nodular, polypoid appearance (Fig. 2). A single layer of ciliated columnar cells surrounded the mass. The tumor cells were large and oval-shaped with relatively small nuclei. Finely dispersed lipoid granules and probably also glycogen were present in the cytoplasm. Figures 3 and 4 show the vacuolated appearance of this tumor.

A benign tumor of epithelial origin was described in the slug, *Limax flavus* L., by Szabó and Szabó (140). In an almost 4-year-old laboratory specimen this whitish, lobated tumor, connected

[1] The quotation is from Prince (108). In spite of considerable effort, in which the assistance of Dr. S. W. Smith (University of Colorado School of Medicine) and of Dr. J. M. Dodd (Gatty Marine Laboratory, St. Andrews University, Scotland) was obtained and greatly appreciated, the original source of this information could not be located. Dr. Dodd kindly supplied us with reference (77).

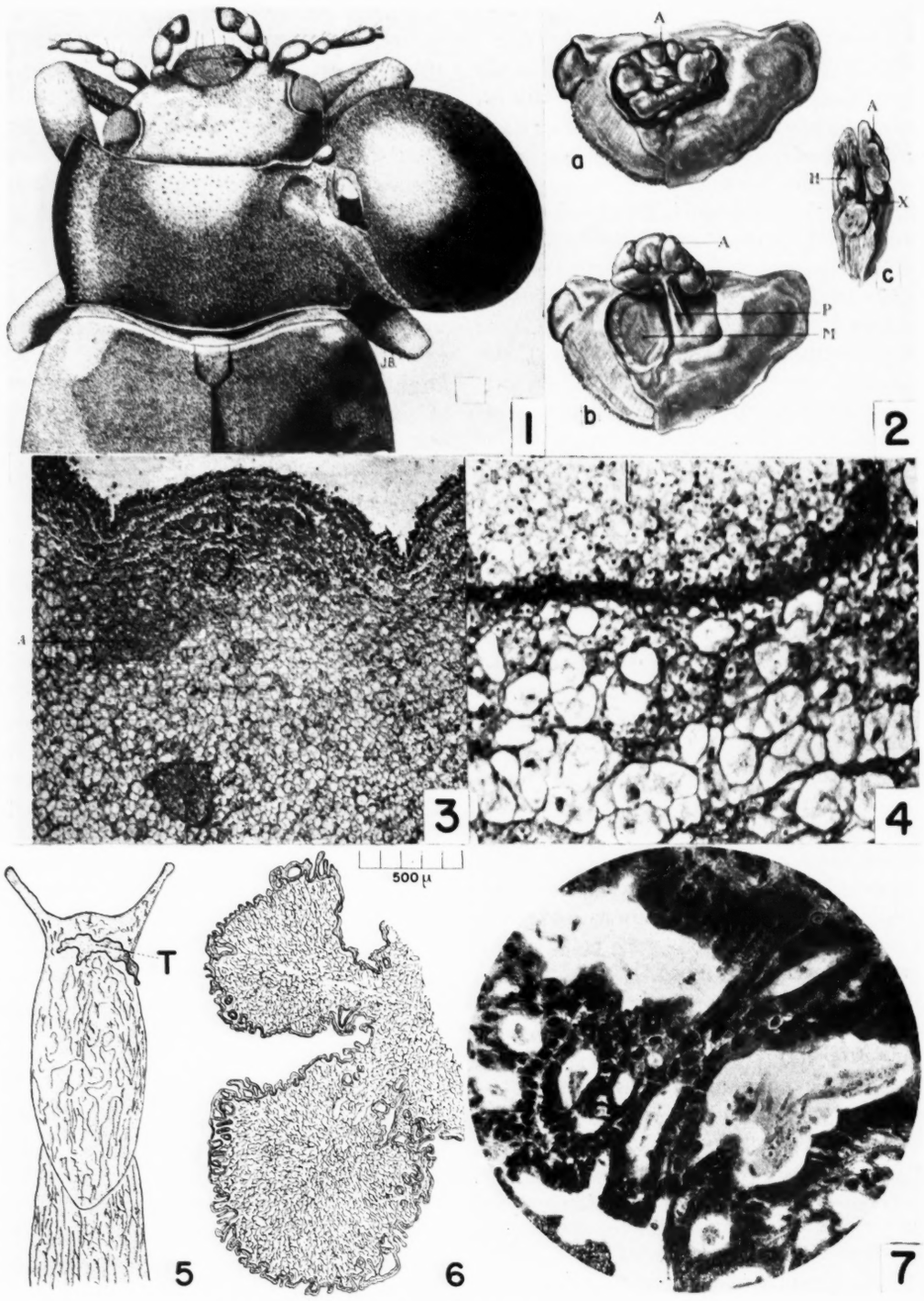


FIG. 1.—Large tumor in prothorax of a beetle (Phytodecta). ×12.5. From Balazuc (4).

FIG. 2.—Oyster (Ostrea) with benign tumor. (*a*) Location of tumor (A) in pericardial region. (*b*) Tumor (A) lifted up, showing pedicle (P) and adductor muscle (M). (*c*) Cross section through tumor (A), showing underlying pericardium (X) and heart (H). From G. M. Smith (127).

FIG. 3.—Section through oyster tumor showing mesenchymal cells and blood vessels (A). From G. M. Smith (127).

FIG. 4.—Same tumor as in Figure 3 at higher magnification. Note large, vacuolated tumor cells with small nuclei. ×75. From G. M. Smith (127).

FIG. 5.—Slug (Limax) showing location of a large spontaneous tumor (T). About life size. Redrawn from Szabó and Szabó (140).

FIG. 6.—Cross section of two lobes which fell off from the tumor shown in Figure 5. Note atypical arrangement of epithelial cells with connective tissue filling each lobe of the tumor. Redrawn from Szabó and Szabó (140).

FIG. 7.—High power photomicrograph of same tumor as shown in Figure 6. Cuboidal epithelial cells forming alveolar tumor tissue; spaces containing some mucus and necrotic cell particles. From Szabó and Szabó (140).

with the border of the mantle by a pedicle (Fig. 5), periodically fell off and grew again. Its maximum size was 15×8×5 mm. Histologically, the epithelial tissue constituting the tumor (Figs. 6, 7) differed from the tissue in normal specimens in that its arrangement was similar to that in alveolar or tubular glands. The center of the tumor mass was filled with dense, richly nucleated connective tissue, whose presence was considered secondary by the authors. The interpretation of this structure as a true tumor is supported by the abnormal histological appearance which set it off from surrounding normal tissues, the tendency for unlimited growth, and the absence of signs of an injury reaction.

Ladreyt (71, 72) found what he considered a malignant growth in a Sipunculus. This abnormal structure had developed in one of the two vascular tubes ("hearts," canals of Poli) which extend along the esophagus of this annelid. Under normal conditions the epithelial lining of these tubes gives rise to various types of blood elements. In the diseased specimen the endo- and perithelium showed extensive cellular proliferation by means of (frequently multipolar) amitoses and nuclear fragmentation. The tumor cells had undergone changes in appearance; they had become fusiform or epithelioid with large, often irregular nuclei. They formed layers around the lumen of the tube which became obstructed in places. Some tumor cells degenerated at the original site, others were disseminated in the blood stream. Musculature, nervous system, and nephridia showed signs of degeneration. The evidence for a diagnosis of malignancy in this case was not considered sufficient by other authors (13, 148). The lack of illustrations in Ladreyt's report further adds to the difficulty of arriving at a conclusive interpretation.

Hereditary factors.—In view of the fact that certain chromosomal deficiencies are known to cause cytological abnormalities which lead to severe morphogenetic disturbances in Drosophila (107), it is not surprising that hereditary "tumors" have been described in this insect. Stark (130), using a strain discovered by Bridges, was the first to call attention to the significance of such tumors in Drosophila. Since then, a number of investigators have worked on Drosophila tumors occurring in various strains. Many important questions concerning the morphology and physiology of these pathological formations are still unanswered, and the fact that not all the investigators were dealing with identical material increases the difficulty of evaluating the descriptions.

According to Stark (130–134) multiple genetic factors in Drosophila larvae of the strain "lethal 7" bring about lethal tumors (Figs. 8 and 9) which occur only in males, and which are comparable to certain vertebrate tumors (melanoepithelioma, lymphosarcoma). These sex-linked tumors arise for the most part in groups of embryonic cells (imaginal discs) which normally give rise to adult organs, but which in this strain become prematurely active (cf. embryonal theory of the origin of tumors). They may also originate in the epithelium of the integument or gut and in "sites of blood formation." As many as fifteen tumors may be observed in one larva; some may grow to one-fourth the size of the host. Excessive amounts of melanin are deposited in the tumors (for chemical tests see 53). They are said to proliferate rapidly, to infiltrate surrounding tissues, to metastasize, and to show mitotic abnormalities. Extirpation of the tumors from larvae prolongs their life but does not permit pupation to occur. Tumor implants in normal larvae cause death of the host before pupation. Larval tumor cell suspensions when injected into adult Drosophila give rise to lethal growths in some specimens. There is evidence that the tumors are not due to the presence of a microörganism.

In addition to this "malignant" type of tumor, Stark described in a mutation of the same strain of Drosophila a nonlethal hereditary tumor, implants of which in normal hosts do not prevent metamorphosis and are carried over into the adult fly. This benign tumor is not sex-linked. It develops at a later larval stage from embryonic cells whose proliferative potency is consequently decreased. These tumors are more limited in growth and become necrotic after being encapsulated by connective tissue elements.

Some authors, such as Needham (88), seem satisfied that Stark's "lethal strain" tumors were indeed malignant. However, many of Stark's interpretations do not appear to be supported by conclusive evidence and have, therefore, met with criticism. The criteria for malignancy seem insufficient (13, 84, 115, 116, 148). Furthermore, Russell (116) claimed that the death of the larvae was caused by an abnormal and extensive disintegration of the midgut cells, permitting the escape of food and thus causing starvation. This abnormality appears a few hours before the first tumors appear. Russell further pointed out that the tumors do not really seem to arise from or be a part of any organ. Whether they are free in the hemocoel or connected with some organ, they look alike and in size most closely resemble groups of amebocytes or of imaginal disc cells. Russell also could find no difference between the malignant and the benign types of tumors. The proliferative growth of tumors and of tumor transplants has not been dem-

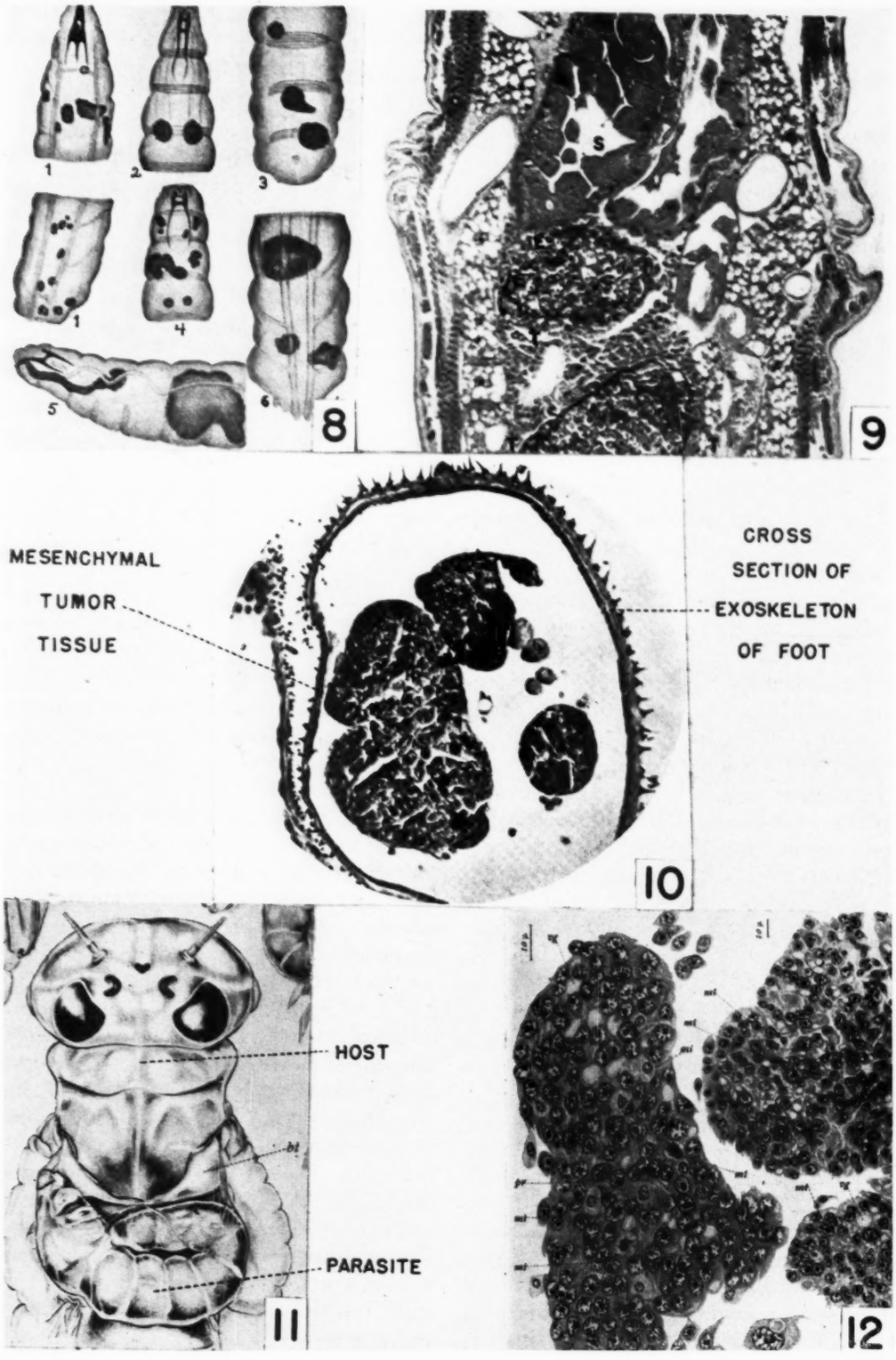


FIG. 8.—Portions of Drosophila larvae with lethal tumors. Dorsal (1 and 6), ventral (2, 3, and 4) and lateral (5) views. From Stark (130).

FIG. 9.—High power photomicrograph of a longitudinal section of a portion of a Drosophila larva showing tumor (T) attached to the wall of the stomach (S). The tumor consists of uniform polygonal cells and contains pigment. From Stark (132).

FIG. 10.—Tumor in the abdominal foot of a lepidopterous larva (Pygaera). From Federley (29).

FIG. 11.—Insect host (ephemerid nymph) with ectoparasitic insect larva (chironomid) causing irritation and subsequent tumor formation; *bt.* = Region of mesothoracic blood sinus pierced by sucking parasite. From Codreanu (13).

FIG. 12.—Syncytial tumor of ephemerid nymph derived from macronucleocytes, developing during "local phase" of proliferative process. Note numerous mitotic figures (mt). From Codreanu (13).

onstrated beyond doubt either by Stark or by Russell.

J. T. Wilson (156) studied two Drosophila tumors, similarly melanotic and dependent on multiple hereditary factors as were those of Stark. In one of the two strains as many as a hundred small tumors were scattered throughout the body, displacing normal tissues. Pigmentation increased with the age of the tumor. In the few survivors the tumor cells disappeared during pupation. The tumors of the second strain were small in number and far less destructive. Histologically, these structures consisted of a center of polygonal cells surrounded by spindle-shaped cells and fibrous noncellular elements which may have originated from blood cells.

In regard to these growths described by Wilson and also those originally described by Stark, the possibility exists that they are encapsulations by blood cells of small tissue fragments, cell agglutinations, or foreign bodies. Such an interpretation is supported by a close study of the published figures (peripheral melanization of the nodules; see 27). The growths described by Wilson were possibly the result of parasitic intrusion from infected food, since tumor incidence was highest in bottles containing relatively few larvae, in which was seen the greatest contamination of the medium by bacteria and fungi.

Morgan, Schultz, and Curry (85, 86) interpreted as "melanotic necrosis" tumor-like abnormalities in the fat body of hybrids of *Drosophila melanogaster* with *Drosophila simulans*, which they attributed to imbalance of heterochromatin between the two species.

The melanotic lesions observed by Gowen (44) in adult Drosophila are different from but show certain parallelisms with the structures reported by Stark.

Tumors of the type originally described by Stark have been studied in regard to relationships between environmental conditions and tumor incidence. Ardashnikov (1) and Russell (117) found the appearance of tumors in Drosophila cultures inversely proportional to the degree of crowding. Temperature is a factor which may modify tumor incidence. According to Hartung (50–52) its effect may be direct (acting on the ontogenetic process) or indirect (affecting other environmental conditions). In general, high temperatures tend to decrease tumor incidence, while lower ones increase it in the three Drosophila strains tested. However, this general statement does not seem to hold for all tumor strains investigated so far. Hartung himself (51) reported an increase in incidence with rising temperature in one of his strains. Similarly, Gardner and Woolf (41) found a rise in incidence from about 76 per cent at 22° C. to about 93 per cent at 30° C. in the growth abnormality "tumorous-head." The first 24 hours of development were determined as a temperature-effective period modifying only one of the two chromosomal factors involved.

Prolongation of the larval period by 2,4-dinitrophenol, or inhibition of larval growth by certain amino acids in high concentration, delay tumor appearance (157; see also 149). 0.1 M arginine added to the basal diet containing sterile brewer's yeast significantly increases the tumor incidence; ornithine does not show this effect (158).

In experiments by Zivin (164) the feeding of thymonucleic acid failed to inhibit the development of Drosophila tumors; the substance had no mutagenic effect in *Drosophila melanogaster*.

The genetic background for the manifestation of these tumors was further discussed by Jones (58) and Russell (117). The tumor incidence may be 100 per cent or less depending on the genetic constitution.

Extensive work is being conducted in the genetics laboratory of the University of Utah (see Gardner, Newby, Dearden, Ratty, and Woolf)[2] on a growth abnormality ("tumorous-head") in *Drosophila melanogaster*. These growths appear on any part of the head derived from the head primordia and are usually external, less frequently internal, and associated with the hypodermis. They are irregular in shape and size, and they seem to be different from the tumors described by Stark. This trait is inherited through the action of a recessive (or slightly dominant) sex-linked gene which produces a maternal effect in the egg and of a semidominant third chromosome gene.

The effect of x-ray treatment on the growth of Drosophila tumors has been studied by several authors with varying results. While Stark (130) noticed no effect, Enzmann and Haskins (26) observed a decrease in the percentage of developing tumors when larvae 18–24 hours of age were exposed to x-rays. Finally, Hartung (49) pointed out that the result depends on the dosage. In a certain strain the hereditary incidence of 15 per cent could be stepped up to 48.3 per cent with 1,500 r. On the other hand, the incidence fell below the control value when 5,000 r were administered. In this connection it is of interest that Pogossiants (105) produced pigmented "tumors" in Drosophila by means of x-rays (4,000 r) which, with respect to morphology and location, resembled the known

[2] We are grateful to Drs. E. J. Gardner and W. W. Newby, University of Utah, for permitting us to report these data while some of the papers were in press.

hereditary types. His results, which he interpreted as "melanotic necrosis (86) give no evidence of malignancy. The structures were not transmitted to later generations (see also 63; for further information on Drosophila tumors see 7, 42).

The hereditary tumors just described are not restricted to Drosophila. In a lepidopterous larva (*Pygaera pigra*, Notodontidae; Fig. 10) similar sex-linked tumors, affecting only the males in the colony while the females are carriers, were observed by Federley (29). To account for this type of inheritance, Federley advanced the interesting hypothesis that the tumors develop from the three polar bodies, which in the male would contain sex chromosomes X, Y, and Y, respectively; in this combination the expression of a recessive gene repeated on each of the Y chromosomes would not be suppressed by the single homologous gene on the X chromosome. Continued division of polar body nuclei is a phenomenon well known in insects.

The tumors which Federley described were of several types. Large numbers of one type floated freely in the hemocoel. Other types were located in various organs, such as hindgut, testes, ganglia, hypodermis, glands, tracheae, and musculature. Vacuolated and giant cells with multipolar divisions, flattened elements, and necrotic structures occurred in some of these tumors. As in the case of the Drosophila tumors, it is uncertain whether these growths in Lepidoptera are true tumors. Since giant cells and multipolar divisions are known to occur normally in the intestinal cells of certain insect larvae (45, 153), and in wound healing (154), more cytological and histological criteria would be necessary for a definite diagnosis of neoplasia in these insects.

In summary, there exist in insects pathological formations which have certain characteristics of hereditary tumors. But despite the relatively large amount of study these growths have received, there is considerable doubt as to their correct interpretation. Various factors contribute to the difficulty. Pigment deposition in the growths proceeds so rapidly that only in the earliest stages can the cells be examined in detail; even there difficulties are encountered in securing adequate fixation (Hartung, personal communication). Therefore, no satisfactory figures or descriptions of the histology of these tumors have been published. No direct evidence has been presented of cell proliferation in the "tumorous" tissue. Comparison with normal or injured tissues is hampered by inadequate knowledge concerning the normal histophysiology of the insect organism. Even in a genus which has received as wide attention as Drosophila, the normal functional relationships of adipose tissue, "lymphocytic tissue," imaginal discs, and free blood cells have been only insufficiently studied (114). In the metamorphosing insect these tissues undergo radical changes which are adequately understood in relatively few species. In view of all these facts it would seem evident that further work must be done before the real nature of these hereditary "tumors" can be clearly established.

Parasitic origin.—Viruses, fungi, microsporidians, and insects parasitizing certain invertebrates have been made responsible for "tumorous growths" observed in their hosts.

In a recent study Bird (5) observed insect tumors associated with a virus infection. The abnormal growths occur in the midgut of the European spruce sawfly, *Gilpinia hercyniae* (Htg.), after infection of the epithelium by the virus. As a result of the infection, polyhedral bodies are formed in the nuclei of the digestive cells of the midgut. In the vicinity of the regenerative nidi abnormal cell proliferations occur which project into the body cavity or, less frequently, infiltrate the cytoplasm of the digestive cells. If larvae become infected just before the last larval molt, i.e., at a time when cellular activity in connection with metamorphosis is at a peak, large tumors develop. Such tumors contain a necrotic, pigmented center, a layer of greatly enlarged infected cells, and an outer region of proliferating cells. During metamorphosis some of these tumors disappear from the gut, either by being pushed into the body cavity or possibly by coming under the influence of digestive juices. The tumor cells do not invade other organs of the infected insects. All available evidence seems to indicate a nonmalignant character of these tumors.

As pointed out earlier, not all abnormal accumulations of cells are tumors. Thus, the proliferative tissue reactions in the fat body of certain insects (Lepidoptera) caused by virus infections (94, 95) probably were the result of an injury reaction (13). Similarly, the "tumeur mycélienne" found by de Boissezon (19) in the abdomen of an adult female of Culex may have been merely a 2-mm. cyst formed of dense fibrous tissue to isolate the fungus present; however, the author stated that the intestinal epithelium in its proximity had undergone morphological changes as a consequence of the presence of the fungus.

Cellular reactions of interest in connection with certain features of tumorous growth have been reported in an ascidian, Ciona, parasitized by the gregarine Monocystis (125, 126). As a rule, almost the entire development of the parasite is intracellular, causing the intestinal cells of the host to react conspicuously. They become enlarged up to 20

times their original size and show a vacuolated cytoplasm. The nuclei hypertrophy and later break down and disintegrate with the rest of the cell. There is evidence that this influence of a foreign organism on the individual host cell is chemical in nature. Up to this point, the observations would seem to indicate cellular hypertrophy due to parasitism, as discussed before. In some cases, however, Siedlecki also noted a reaction in neighboring cells; the epithelium proliferated, and even connective tissue elements became involved in the resulting abnormal growth. These tumor-like structures, in which the original cause, i.e. the parasite, is often no longer found, were compared by the author with mammalian liver adenomas.

Papilloma-like structures, of mesodermal origin, have been described in the annelid worm *Potamilla torelli* by Mesnil and Caullery (81) as caused by another sporozoan parasite. They grow rather intensely, ramify, and protrude into the coelom, which is filled with parasites. In these growths the nuclei continue to divide amitotically, even after the parasites have disappeared. It is difficult to decide whether or not these structures should be classified as tumors, or whether they are simply hyperplastic formations.

Several authors have studied the effect of insect parasites on insect hosts (98, 99), but the most informative results are those of Codreanu (12, 13). An ectoparasitic larva, the chironomid Symbiocladius, feeds on the blood of certain insect hosts, i.e. ephemerid nymphs of the genera Heptagenia, Rithrogena, etc. (Fig. 11). When so doing the parasite causes the following progressive reactions in the host. Aside from an injury reaction due to irritation, a proliferative process takes place which consists of two phases. During the "local phase" a syncytial tumor derived from macronucleocytes but gradually changing in its histological appearance (increasing cytoplasm, vacuolization, change of nuclear and nucleolar size, abnormal mitoses) appears near the site of the parasite (Fig. 12). In the "generalized phase" which follows, free macronucleocytes accumulate in the circulation (leukemia), infiltrate organs of the host, and form small nests, for instance in the vicinity of the ovarian tubes. The formation of these tumors which Codreanu compares with a leucosarcoma, and which in all probability are malignant, continues even when the ectoparasite is removed at an early stage. The host invariably dies before the completion of its development with tissues depleted and prematurely "aged."

Experimental work.—In the investigation of vertebrate and invertebrate tumors alike the most useful material is that in which neoplastic growth can be produced experimentally. Various attempts have been made to induce tumors in invertebrates in which either the customary ways of approach, such as the administration of carcinogenic hydrocarbons, or essentially new methods have been used. The effects of chemicals, of bacteria, of endocrine and of nervous factors have been studied.

Hammett and his associates conducted extensive investigations in an attempt to elucidate growth mechanisms relative to neoplasia. Experiments with various invertebrates, as well as with plants, mice, and humans led these authors to postulate that cell proliferation with consequent growth is stimulated and regulated by certain chemical compounds containing the sulfhydryl group. In the course of regeneration experiments on the hermit crab, *Pagurus longicarpus*, accidental injuries often occurred at the site of a regenerating chela and between segments of the new chela where the chitin was thinnest (47). Aberrant growths developed from such lesions in controls as well as in animals treated with *p*-thiocresol. However, in the experimental group all lesions resulted in growths, whereas only 75 per cent of the controls produced growths. Furthermore, the growths in the group treated with the chemical were always considerably larger. In a few instances, and only after treatment with *p*-thiocresol, crabs developed an aberrant growth on the tip of the regenerating chela in the absence of a lesion. Histologically such aberrant growths consisted of normal tissues in a highly unorganized arrangement showing no resemblance to any organ. In the opinion of the authors, these pathological overgrowths can be compared with malignant tumors. Figure 21, p. 348, of the paper by Hammett and Hammett (47) may serve as an example. It shows a section through a growth which had developed "spontaneously," i.e. without injury, after *p*-thiocresol treatment at a site which is normally an intensely proliferating center. It is described as consisting of epithelial cells without chitin formation and is interpreted as a wildly disorganized proliferation analogous to certain types of malignancy. The magnification at which the photograph is reproduced does not permit the distinction of cell types, and further details about the histology of this growth and the ultimate fate of similar structures are not given. From a study of the material presented one gets the impression of an injury reaction at a rather early stage. More information would seem necessary for an evaluation of the possible malignant potencies of this and similar structures.

Bacterium tumefaciens, known to cause tumors in plants and in fishes, was inoculated into the marine annelid Nereis by Thomas (145–148). The

conditions under which tumorous growth took place in these experiments are of interest because, in addition to the bacterial action, an ecologic factor is involved. The author found that in a given habitat, characterized by a certain salt content of the water, the oöcytes of the worms degenerated and gave rise to granulomas (88). Only specimens thus affected responded to the stimulus of bacterial

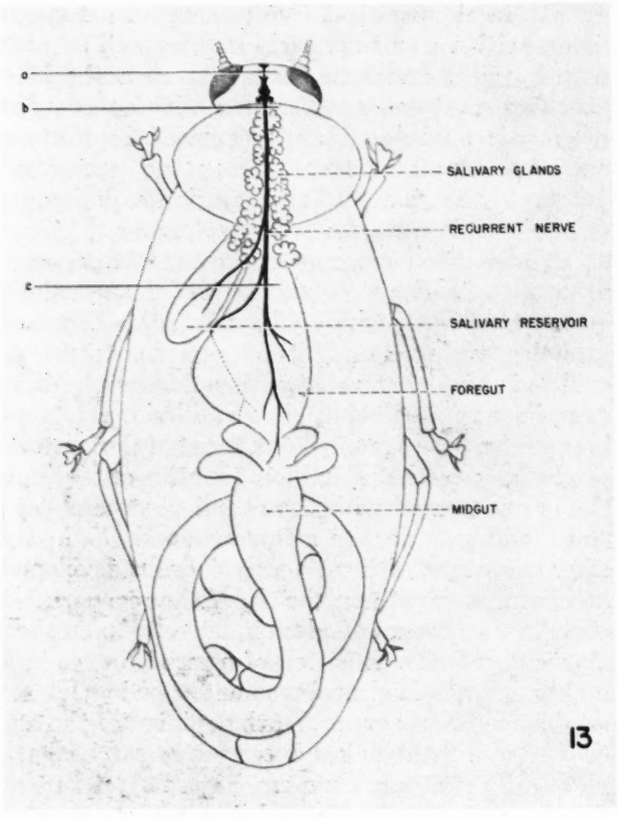


FIG. 13.—Diagram of Leucophaea, showing sites of tumor formation, i.e., anterior portion of alimentary canal (foregut and midgut) and salivary organs (salivary reservoir and glands), and their innervation by branches of the recurrent nerve. *a*, *b*, *c*, three locations where nerve has been cut. ×2. Orig.

infection with abnormal growth of the connective tissue, appearance of giant nuclei, atypical mitoses, whorl formation, etc. The resulting tumors were characterized by fast growth and invasiveness. They were fatal to the worm and were "sarcomatous" in appearance. Thomas, therefore, considered them as malignant or close to malignant growths, an interpretation which was subsequently questioned by Codreanu (13).

Earlier attempts to cause tumors in caterpillars of *Galleria mellonella* by inoculation of *Bacterium tumefaciens* emulsions were unsuccessful (66).

Another approach to induce tumor growth in invertebrates, chosen by several investigators, consisted of the introduction of foreign bodies, irritants, etc., into experimental animals (p. 404). Implants of this kind frequently caused injury reaction and cyst formation by fibroblasts. In the experiments of Labbé (70) who used celloidin soaked in coal tar in the marine snail Doris, tumor-like structures subsequently developed. Their cells, being fibroblastic in origin, became giant, plurinuclear, their growth pattern invasive. While Labbé himself took these criteria as signs of a cancer-like behavior, Thomas (147, 148) could not produce comparable effects in Nereis, Ascidia, and other forms and, therefore, expressed a certain skepticism with regard to Labbé's conclusions.

During the last decade, newer methods were adopted in the application of carcinogenic compounds. The carcinogens used in invertebrates were the common ones, methylcholanthrene, 1,2,5,6-dibenzanthracene, benzpyrene, etc. They were administered by mouth, as implants of crystals, as aerosols in the surrounding atmosphere, or dissolved in aqueous media, etc.

A response most closely approaching those observed in mammals due to the action of carcinogenic hydrocarbons was elicited in cephalopods (Sepia) with 1,2,5,6-dibenzanthracene (57). Within a few days after the subcutaneous administration of a pellet of this compound a whitish tumor, several cm. in diameter, could be observed. Histologically, the epithelium and subcutaneous connective tissue appeared replaced by numerous diffusely infiltrating histiocyte-like cells which showed signs of degeneration (autolysis; homogeneous, pyknotic nuclei). There was no indication that the tumor was attacked by the defense mechanisms available to the host. This type of lesion differs essentially from the injury reaction observed earlier in the same species, as well as in Octopus and Eledone, as a consequence of trauma or irritants (59–61) or of subcutaneous coal tar injections (62).

The tolerance for carcinogenic azo dyes in the cockroach, Blattella, is much higher than in the rat or mouse. After administration of 0.2 per cent of three of these dyes in the diet nodular formations were observed in these insects, whose tumorous nature could, however, not be established (90).[3]

Based on the hypothesis that cancerous growth may be correlated with mutations occurring in somatic cells, the effect of carcinogens on the mutation rate of Drosophila was studied. While

[3] The effect of benzpyrene on insects is currently being studied by M. P. Boulet, Musée zoologique, Université de Strasbourg, France, to whom we are indebted for this information.

Auerbach (2) had negative results, Demerec (21, 22), using a different technic, was able to produce gene mutations (considerable increase in lethal mutations) with four carcinogenic hydrocarbons, as well as with a nitrogen mustard (3). The mutagenic effect of these compounds, especially of dibenzanthracene, on Drosophila is very similar to that of x-rays, ultraviolet light, and neutrons—all agents whose carcinogenic capacity is well known.

Phenol has a positive effect on the mutation rate of Drosophila (46), an observation which is of interest in view of clinical evidence that this compound may produce skin cancer (138, p. 165).

The early phase of growth, but not cell differentiation, was stimulated in the marine hydroid Obelia by 1,2,5,6-dibenzanthracene and by methylcholanthrene in experiments by Hammett and Reimann (48) and Reimann and Hammett (113). Since cell proliferation is one of the primary manifestations of cancer, these results have some bearing on the problem of neoplastic growth.

Regeneration in planarian worms was stimulated by the presence of carcinogenic compounds without effect on the histological appearance of the tissues (93).

Tchakhotine (143) exposed sea urchin eggs to sodium monobromo- or monoiodoacetate with the result that atypical tissue proliferation took place. The mesenchyme became "wild," filling up the blastulae and thus preventing gastrulation. The outcome was fatal in the early sea urchin stages.

In a study involving a number of chemical substances Rapaport (110) found that arsenic and boron compounds caused melanotic tumors in Drosophila larvae, similar to the hereditary types already discussed.

The effect of carcinogenic hydrocarbons on protozoans has been studied by several authors. Tittler (150), using pure cultures of a ciliate (Tetrahymena) was unable to detect mutagenic effects with 1,2,5,6-dibenzanthracene, 3,4-benzpyrene, and methylcholanthrene in concentrations ranging from 1:50,000 to 1:200,000. This observation is in contrast to the positive results in Paramecium reported in earlier papers (87, 129, 151, 160). The effects observed in these experiments (polymorphism, increased growth rate, enhanced vital functions) may have been indirect, i.e. they may have been brought about via the bacteria, present in the cultures on which Paramecium feeds. This interpretation suggested by Tittler would seem plausible, because carcinogenic hydrocarbons are known to increase the rate of reproduction of bacteria, such as Escherichia (43, 55). However, before a more definite statement can be made, experiments with bacteria-free cultures should be extended to include other ciliates, especially paramecia.

In view of the known connection between endocrines and tumor growth in mammals it is significant that abnormal tissue reactions similar to certain vertebrate tumors could be elicited in the insect Carausius (Dixippus, walking stick) by Pflugfelder (100, 103, 104) by means of an experimentally induced disturbance of the hormone balance in early nymphal stages. This effect, as well as degenerative processes, was brought about by the removal of the corpora allata. Reimplantation of these endocrine glands prevented the abnormal tissue reactions. Implants of supernumerary corpora allata also caused abnormal growths. The atypical growths were observed in certain locations, especially in mesodermal structures. Amitotic divisions were noted in the proliferating part of

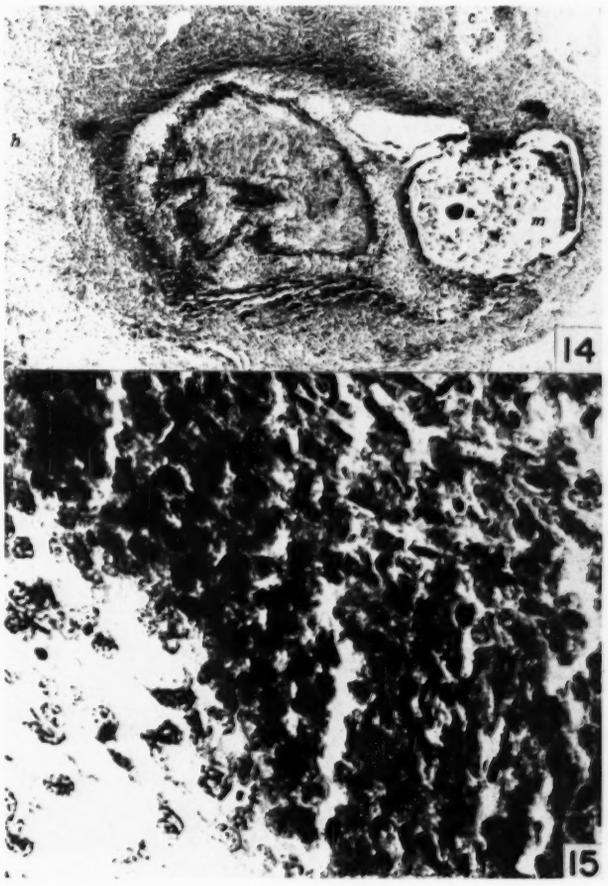
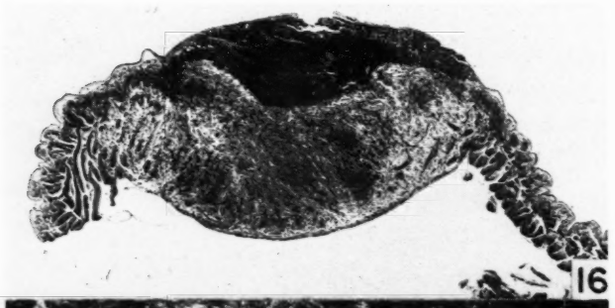


FIG. 14.—Midgut tumor of Leucophaea. The large tumor mass, replacing the normally thin gut wall incorporates parts of the intestinal caeca (*c*), and the hindgut (*h*). *m*, lumen of midgut with debris. About ×24. Orig.

FIG. 15.—Same tumor as in Figure 14 at higher magnification. The tumor cells differ greatly from the cells normally composing the midgut wall. ×360. Orig.

the gut at the place of origin of the Malpighian vessels. The wall of the oviduct and the corpora cardiaca showed similar changes with occasional giant nuclei.

Atypical new growths could also be produced when embryonic tissue of the same insect species was transplanted into the head capsule of nymphs or adults whose own corpora allata had either been



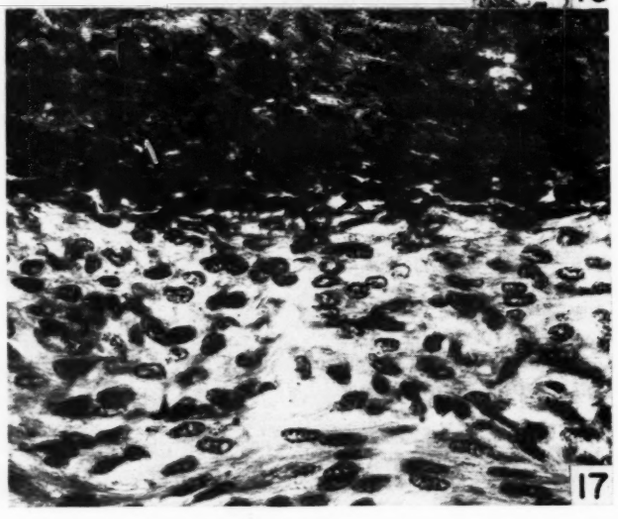


FIG. 16.—Tumor in wall of foregut of Leucophaea. To the left and right of the tumor the wall is normal, consisting of a muscularis and a chitin-covered epithelium facing the lumen (above). Note stratification of the tumor. About ×24. Orig.

FIG. 17.—Tumor tissue of Figure 16 at transition of layers at higher magnification. The types of tissue shown do not normally occur in the foregut wall. ×360. Orig.

extirpated or which had received supernumerary allatum implants (101, 103). The abnormal growth of the embryonic tissue, taking place in part by mitotic division, in part by nuclear fragmentation (atypical amitoses), involved all three germ layers. Certain neuroblasts, for example, developed no axons, became giant elements, while their nuclei divided amitotically. Glia cells surrounded such pathological elements and proliferated to form cystlike structures or large cell aggregates. The prospective midgut (entoderm) showed profound abnormalities with cell proliferation by means of mitotic and amitotic nuclear division. The embryonic mesoderm displayed a tendency to invasive growth which affected particularly the muscles of the host. No pathological growths of this kind were observed in embryonic implants which had been transferred to normal hosts serving as controls. Spemann (128) has discussed the production of tumors by embryonic tissue implants in vertebrate hosts. It is difficult to state at this point to what extent the abnormal growths in Carausius are comparable to true neoplasms, but the fact that they are definitely due to an endocrine imbalance should make them interesting objects for further study.

An essentially new way of inducing tumors was found in Leucophaea, a large roach which offers many advantages for experimentation (119, 120, 122). In this insect the severance of a nerve (recurrent nerve) causes tumors to develop in organs which are innervated by this nerve. The organs concerned are the anterior portion of the alimentary canal (foregut and anterior midgut) and the salivary organs (salivary reservoir and salivary glands). Figure 13 shows the topography of these organs and their innervation by branches from the recurrent nerve. Three possible locations for nerve transection are indicated; all three types of operation were carried out with essentially the same results. The tumors developing in a large proportion (75–80 per cent) of the operated adults and nymphs after varying periods of time were, as a rule, conspicuous structures. Histologically, they consist of layers of cells which in the course of development apparently became progressively more abnormal and finally necrotic. Few, if any, mitotic figures were observed, a fact which, in view of the often rapid growth of the tumors, may be explained by assuming that the mitoses are completed within a very short time or that cell division is mainly by amitosis. Giant cells, not normally observed in the organs in question, are not infrequent in the tumors.

The most commonly affected organ is the anterior portion of the midgut (stomach). Normally consisting of an almost transparent columnar epithelium and muscularis, the mucosa increases in width many times after nerve section and becomes opaque or brown (Figs. 14, 15). Severe tumors of this kind usually lead to the death of the animal. A foregut tumor, sarcoma-like in its microscopic structure, is shown in Figures 16 and 17. The conspicuous tumors of the salivary reservoir (Figs. 18, 19), which is normally a thin transparent membrane, suggest epithelial tumors. Their size may become impressive (diameter up to 10 mm.).

The tumors of a certain number of operated insects showed signs of malignancy (121). Tissues near the original tumor site were invaded and sometimes incorporated in a large mass of tumor tissue. In a few cases this process led to the perforation of the chitinous body wall.

The primary (possibly degenerative) effect of severance of the recurrent nerve on the tissues which it innervates, and the early phases of the pathological growth process which this operation brings about, are not yet fully understood. The possible role of connective tissue and blood elements in this process need further study. Insects are capable of producing large masses of connective tissue at sites of injury. The tumors in Leucophaea, which develop at locations distant from the injury caused by the operation, do not resemble such connective tissue masses. Control operations with equal or greater injury at the same site, but without nerve severance, do not lead to tumors. The tumors due to nerve severance do not show melanization. Giant cells occur in certain insects normally, or during wound healing, but the ones mentioned in the Leucophaea tumors are evidently not involved in the healing process, are different from those observed in cases of injury reaction, and are normally not found in the corresponding tissues of nontumorous specimens. The histological appearance of the tumors developing in organs whose innervation has been disturbed by severance of the recurrent nerve differs to such an extent from the normal histology of the organs in question that these tumors cannot be looked upon merely as the result of tissue hyperplasia. Against their interpretation as ulcerative processes speak the lack of necrosis and the maintenance of surface continuity in the earlier phases of their development.

Following severance of the recurrent nerve in Leucophaea early death from the resulting tumors is more frequent in females than in males. In animals which have been gonadectomized several weeks previous to nerve severance this sex-linked difference in mortality is no longer observed. The survival rates in tumor-bearing castrates of either sex are approximately equal and represent values intermediate between those of males and females in which only the nerve has been cut (123). These differences may perhaps be based on sex differences in metabolism, in that the greater lability in the metabolic pattern of the reproducing females may account for their lower resistance to tumor growth. In an attempt to substantiate these possible correlations, the fat content, in relation to total dry matter and body weight, of whole normal, castrate, and tumor-bearing animals was compared (159). The content in body fat of the tumor-bearing males (with nerve severance only, or with nerve severance after castration) was below normal and close to starvation levels. The corresponding values for females with nerve severance ranged from starvation to normal levels. The amount of fat in ovariectomized females with tumors ranged from the low values found in starvation to the above average amounts characteristic of castration. These data seem to indicate essential differences between males and females. In at least some of the analyzed females death cannot be attributed to starvation in connection with tumorous changes in the alimentary canal, since it occurred at a time when the fat reserves were not yet diminished.

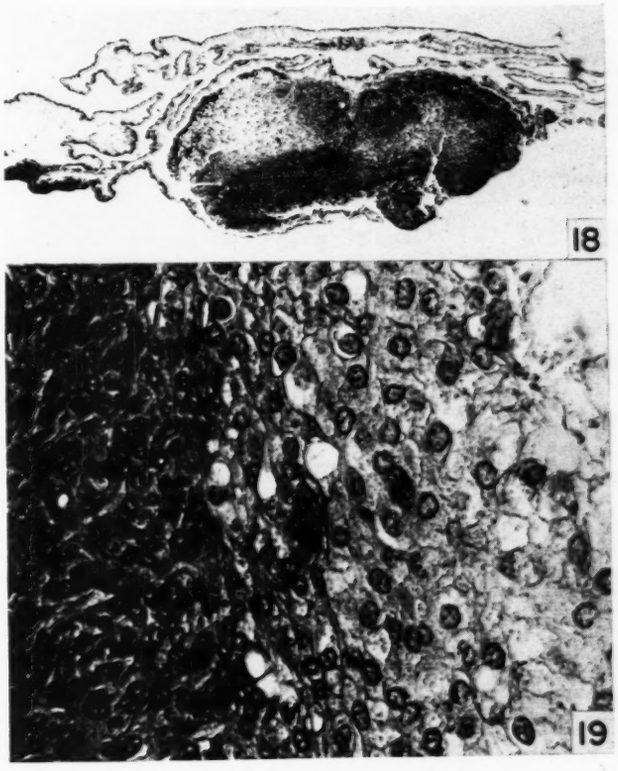


FIG. 18.—Tumor in the wall of the salivary reservoir of Leucophaea, filling part of the lumen. About ×24. Orig.

FIG. 19.—Salivary reservoir tumor of Leucophaea at higher magnification. ×360. Orig.

SUMMARY

Phenomena related to neoplastic growth have been studied in a number of invertebrate phyla, and tumors of either epithelial or connective tissue origin have been reported in annelids, sipunculids, arthropods, molluscs, and ascidians. Evidence of the true neoplastic character of such alleged tu-

mors is often scanty; reasonably convincing signs of malignancy are found only in a small number of reported cases.

A number of tumors described in representatives from various invertebrate groups may be classified as spontaneous growths, since their cause is unknown. In insects certain of the alleged tumors are known to be hereditary. Other factors reported to cause tumors or abnormal growths in invertebrates are parasites, endocrine imbalance, and disturbance of innervation. Carcinogens have been tried on a variety of invertebrates and have been reported to cause tumors only in molluscs. X-rays in suitable dosage are known to cause tumor-like growths or increase their incidence in insects (Drosophila).

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Response of a Transplantable Lymphosarcoma to Colchicine

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Many attempts have been made to cure experimental tumors or human malignant growths with colchicine. In 1941, Ludford (1) concluded that colchicine produces "complete regression of some tumors, but only by employing large doses of the drug just below the minimum lethal dose." Lits, Kirschbaum, and Strong (2), using a transplantable lymphosarcoma in mice, found that colchicine would apparently cause complete regression in some animals but that recurrence inevitably occurred. One of the most successful attempts in treating a transplanted lymphosarcoma was reported by Heilman and Kendall (3). They showed that Compound E (11-dehydro-17-hydroxycorticosterone) produced complete regression in some mice bearing a transplantable lymphosarcoma; however, in most animals regression was not complete or if complete was only temporary. Bass and Feigelson (4) demonstrated that colchicine is more effective than nitrogen mustard or urethan in producing acute regression of 6C3HED tumors in mice. In these studies, tumor regression was invariably observed following colchicine administration. The object of the present study was to extend these observations and to investigate the effects of prolonged administration of colchicine on this tumor.

MATERIALS AND METHODS

A 6C3HED lymphosarcoma originally obtained from Dr. W. U. Gardner was used. This tumor has been carried through repeated transplantations in our laboratory for the past 3 years. It does not regress spontaneously, and only rarely is a transplantation unsuccessful. It is, therefore, highly satisfactory for studying chemotherapeutic agents. Without therapy the tumor-bearing mice live 18–21 days. All animals were fed Purina Dog Chow and given water *ad libitum*. C3H or C3H F_1 hybrid mice 6–12 weeks old were used. Initial tumor transplantation was made with a 15-gauge needle. Small pieces of tumor were placed subcutaneously in the region of the right axilla. Tumor size was measured at frequent intervals.

Colchicine U.S.P. dissolved in distilled water was administered intraperitoneally. Treatment (unless otherwise stated) was begun when the tumor measured approximately 1 cm. in diameter (8–14 days after implantation). For comparison, ethyl carbamate and cortisone were also employed. Ethyl carbamate (urethan) was administered intraperitoneally, cortisone (cortisone acetate in saline suspension), subcutaneously.

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RESULTS

The results obtained are shown in Table 1.

When tumor-bearing C3H F_1 hybrid mice were treated with colchicine (0.75 mg/kg) daily for a period of 23 days (Group 1), the transplanted tumors steadily regressed until they entirely disappeared in from 4 to 9 days. Animals so treated were observed for a period of 150 days without showing evidence of recurrence. These animals were then the recipients of a second transplant, made in the region of the original implant. In a few instances, small nodules developed which rapidly regressed; their nature was not investigated. A third transplant was then made on the left side, opposite the site of the original transplant, following which there was no evidence of tumor growth.

In a group of nine mice receiving a smaller dose of colchicine (0.5 mg/kg) for the same period of time, complete regression of the implanted tumor occurred in four animals. Reinoculation at the site of the original implant resulted in the appearance on about the eighth day of small nodules which entirely disappeared by the thirteenth day. When the transplantation was repeated on the opposite side of each animal, no evidence of a take was observed.

A third group of ten animals was selected for a shorter period of therapy. The animals were given a daily colchicine dose of 0.5 mg/kg which was increased to 0.75 mg/kg if the tumor failed to regress. The total period of treatment varied from 5 to 9 days. All tumors regressed. On the 94th day, and again on the 124th day, after the initial transplantation attempts to obtain new takes of the same tumor with the procedure described in the preceding paragraph were unsuccessful.

To a fourth group of twenty C3H mice colchicine (0.75 mg/kg) was administered daily for 3 days. In twelve animals the tumor showed complete regression; these animals were not receptive to the tumor 46 days following the original implantation.

To exclude the possibility that the age of the animal might be a factor in the failures obtained on the second and third transplantations, fresh tumor fragments were implanted in the right axilla of six 7-month-old normal mice. Takes were obtained in all six animals.

The results from a 2-day course of therapy are shown in Table 1, Group 5. Out of 28 C3H F_1 hybrid mice so treated, ten tumors regressed and did not recur during a 5-month period of observation.

DISCUSSION

Immunity following chemical cure of a tumor with colchicine has been reported by Peyron, Lafay, and Kobozieff (5), who used the Shope rabbit papilloma. Cured rabbits were immune to the virus. The development of antibodies in mouse leukemia has been reported by Furth (6) and Gorer (7). The recent observations of Stoerk and Emerson (8) show that riboflavin deficiency causes permanent suppression of the 6C3HED tumors associated with the development of immunity, whereas pyridoxine-deficient animals show tumor suppression only while the therapy is continued. This is interpreted by the authors as a failure of immunity to develop under the conditions of the latter experiment.

TABLE 1

RESPONSE OF 6C3HED TUMORS TO COLCHICINE THERAPY

Group exp.	Treatment	Strain of mice	Permanent complete regression	Treatment deaths
1	colchicine 0.75 mg/kg daily for 23 days	C3H F_1 hybrid	8/10	2/10
2	colchicine 0.50 mg/kg daily for 23 days	C3H F_1 hybrid	4/9	0/9
3	colchicine 0.5 mg/kg to 0.75 mg/kg daily for 5–9 days, varying with tumor response	C3H F_1 hybrid	10/10	0/10
4	colchicine 0.75 mg/kg daily for 3 days	C3H	12/20	3/20
5	colchicine 0.75 mg/kg daily for 2 days	C3H F_1 hybrid	10/28	1/28
6	none	C3H*	0/20	
7	none	C3H F_1†	0/6	
8	urethan 100 mg/100 gm body weight daily 8 days	C3H	1/18	9/18
9	cortisone 2 mg. daily for 4 days	C3H	0/8	5/8

* Mice were approximately 2 months of age at time of transplantation. Mice were all dead by 22d day.
† Mice were approximately 7 months of age at time of transplantation. Mice were all dead by 22d day.

For comparison, the responses to urethan and cortisone administration are included. No permanent regression was obtained with cortisone in doses which were definitely toxic; one permanent regression was observed with urethan.

Since it had been shown that permanent regression was obtained with colchicine treatment (Table 1), an attempt was made to produce a tumor resistant to treatment by giving the drug so that partial regression would be repeatedly obtained. Although no resistant tumors were obtained by this method, additional evidence of permanent regression was obtained. These tumors were allowed to grow for 15 days from the time of implantation and they measured 1.3–1.5 cm. in diameter at the institution of therapy. Seventy-nine tumor-bearing animals were used. Colchicine was found to be more toxic to those animals with tumors which were larger at the beginning of therapy. The tumors in 27 animals regressed completely. Although our data would indicate that better results are obtained with early treatment, a possible influence of the sex of the animals cannot be excluded without further study. All surviving animals have been observed for a minimum period of 5 months.

The present results indicate that 6C3HED tumors in C3H mice regress completely and apparently permanently when the animals are treated with appropriate doses of colchicine. Although the number of treated animals studied in each group is not large, the results appear significant, since none of the untreated controls regressed. Moreover, our experience with the tumor in the past 4 years has shown that no tumor regression has occurred in healthy animals. It is possible that the difference in results here reported from those of Lits, Kirschbaum, and Strong (2) may occur because of the treatment schedule employed, the strain of mouse used, or some specific characteristic of the tumor. The failure of cortisone, under the conditions of this experiment, to produce permanent regression would strongly suggest that the "tumorolytic" action of colchicine is not on the basis of an "alarm reaction" but rather a direct effect of the agent on tumor tissue.

CONCLUSIONS

It has been shown that colchicine administration produces permanent regression of lymphoma 6C3HED. Evidence is presented which strongly

indicates that the colchicine acts directly on the malignant lymphoid tissue.

An immunity to this tumor developed after regression of the initial implant. This immunity developed before the 46th day and persisted to at least the 173d day after original transplantation was made.

ACKNOWLEDGMENT

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Studies in Hodgkin's Syndrome

X. The Morphology and Growth Patterns of Explant Cells Cultivated *in Vitro**

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Although the morphology and growth patterns of Hodgkin's disease explant cells in tissue culture have been the subject of a number of reports—Mankin (6), Meier (7), Lewis (5), Grand (3), and Rottino (9, 10)—attempts to subdivide and classify explants representing individual patients have been based on differences said to reflect the "stage of the disease" at the time of biopsy (6, 3). These differences consist primarily of changes in the relative proportion of the specific cell types which appear initially in the zone of growth.

Mankin (6) has observed that the number of lymphocytes present in the growth ring following explantation and their relative resistance to degeneration are greater early in the disease than later. A similar observation is recorded by Grand (3), who notes that explants which are considered to represent "early pathology" contain a predominance of lymphocytes and that advance in "pathology" is accompanied by a greatly diminished number of lymphocytes.

Hodgkin's disease is considered by Mankin (6) to represent an abnormality of the reticular stroma in which there is a failure of differentiation into typical connective tissue cells and fibrous intercellular substance. Tonofibrils appear later than normal, reticular cells are present in greater than normal numbers, vary widely in size, shape, and appearance, and are unable to evolve toward network forms. Meier (7), in describing the Hodgkin's explant, refers to a more rapid initial growth and migration of cells than normal and to the variable appearance of cultures depending upon whether "small or larger" cells (of endothelial origin) predominate. In the presence of "moderately advanced pathology," Grand (3) observes an increase in the number of reticulum cells, compared with relatively few of these cells in explants representing "early pathology," and their replacement by spindle-shaped fibroblasts in explants representing "late pathology."

Although the presence of fat bodies in diseased reticular cells is mentioned by Mankin (6), no comparison with normal cells is made. Meier (7) qualifies his observations in this respect by reporting that, although the "large round cells" which predominate in some cases may contain strongly granulated cytoplasm, a number of cultures contain only optically homogeneous cells.

Large, multinucleated giant cells which are more or less flattened, with an endoplasmic structure which stains intensively in contrast to the clear, foam or gauze-like ectoplasmic structure, may contain 100 or more nuclei (6). Mankin (6) and Grand (3) have referred to the fact that these cells are observed only in Hodgkin's disease and Meier (7) to the unequal size of nuclei in Hodgkin's giant cells. Mankin states that both the abnormal reticular cell and the giant cell of Hodgkin's disease arise from the same cell progenitor, the reticular cell, and Meier that the affected cell in Hodgkin's disease originates in endothelium. Lewis (5), in a series of motion picture studies, observes that the "small Dorothy Reed cells," some of which are identical in appearance with the myeloblast, migrate freely with a writhing motion. The "large Dorothy Reed cells" are sluggish and nonphagocytic and resemble, but are not identical with, the "megalokaryocyte." The author concludes that Dorothy Reed cells are of myeloid origin, resembling most closely the "malignant myeloblast." Rottino (10) classifies the Hodgkin's giant cell as a foreign body-type cell and states that it arises independently of the original Sternberg-Reed cells present in the explant.

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Meier (7) considers that the connective tissue of Hodgkin's disease is unusual since, when first explanted, it stretches out in large loose folds around the explant. After single or repeated transfers, its growth begins to extend radially from the explant rather than irregularly around it. With repeated transfer, closely packed, small and thin connective tissue cells, not unlike those of normal lymph nodes, growing radially from the explant become the predominant growth element at the expense of other cell forms present in the original culture. Lewis (5) observes that lymphocytes, Dorothy Reed cells, and stellate macrophages, which grow out in the early cultures and their subcultures are soon overgrown by a "luxuriant out-growth of giant stroma" cells containing large single or multilobed granular nuclei with large nucleoli. Tripolar and bipolar mitotic figures with an increased number of chromosomes are present. Grand (3) describes the "spindle-shaped fibroblast" as the predominant cell in cases representing "late pathology"; lymphocytes, Sternberg-Reed cells, and fibroblasts are said to make up the cell population during "early" and "moderately advanced pathology."

Grand (3) reports the demonstration of intracytoplasmic inclusion bodies in cultured reticular cells, lymphocytes, and fibroblasts, using brilliant cresyl blue and Seller's stain. He also states that liquefaction and disintegration are a prominent feature of cultures representing both "early" and "moderately advanced pathology." Rottino (9, 10), on the other hand, concludes that, although giant cells, liquefaction and disintegration of cultures, and inclusion bodies may be more frequent, they are not specific for Hodgkin's disease in tissue culture. He considers inclusion bodies to be due to ingestion of fragments of dead lymphocytes, suggests that the diseased reticulum cell may give rise to the enzyme responsible for liquefaction, and finds it impossible to correlate morphologic or other characteristics of the cultured explant with the degree of liquefaction observed.

An attempt will be made in the following discussion to separate explant types in Hodgkin's disease into two categories. It has been observed in this laboratory that not all explants obtained from Hodgkin's disease patients appear to have the same morphologic and growth pattern characteristics in tissue culture. The significance of these differences will be discussed.

MATERIALS AND METHODS

Human lymph nodes were obtained under sterile conditions at biopsy. Part of each specimen was subjected to histologic examination by three pathologists. The remainder was cultured within 4 hours after removal, using the roller-tube tissue culture technic (2). A balanced salt solution, Gey's solution, was sterilized by filtration and added to the nutrient fluid. Bovine thrombin and bovine fibrinogen (8), obtained commercially in powdered form, were used routinely as a clotting medium in all tissue cultures.

Human placental serum and human adult serum (cell-free) were routinely used as a part of the nutrient fluid. Placental blood was obtained from the cord vein following delivery of the placenta. After clotting, the serum was removed and stored at 4° C. until used. Adult blood was obtained by venipuncture and its serum prepared and stored in the same manner.

Fifty lymph node tissues, subsequently identified as Hodgkin's disease by examination of fixed and stained sections of the original tissues, were explanted and classified as Types I, Ia and II (see Results).

The roller-tube technic (2) was employed in culturing tissues. The tissues were minced with scissors, and six pieces, 0.5 mm.–1.0 mm. in size, were

Figures 1–8. All cultures illustrated were living and unstained when photographed

FIG. 1.—Hodgkin's lymph node, Type I. The presence of macrophage-type cells crowded with fat bodies and free cell growth are illustrated. The cell types indicated by arrows are: (A) the macrophage-type cell and (B) the lymphocyte. 17-day culture. Mag. × 300.

FIG. 2.—Hodgkin's lymph node, Type I. Macrophage-type cells and reticulum cells crowded with fat bodies in association with free growing fibroblasts. The cell types indicated by arrows are: (A) the macrophage-type cell, (B) the reticulum cell, and (C) the fibroblast. 7-day culture. Mag. × 240.

FIG. 3.—Hodgkin's lymph node, Type I. The same type of growth as Figs. 1 and 2 (at higher magnification). The cell types indicated by arrows are: (A) the macrophage-type cell and (B) the reticulum cell. 6-day culture. Mag. × 1500.

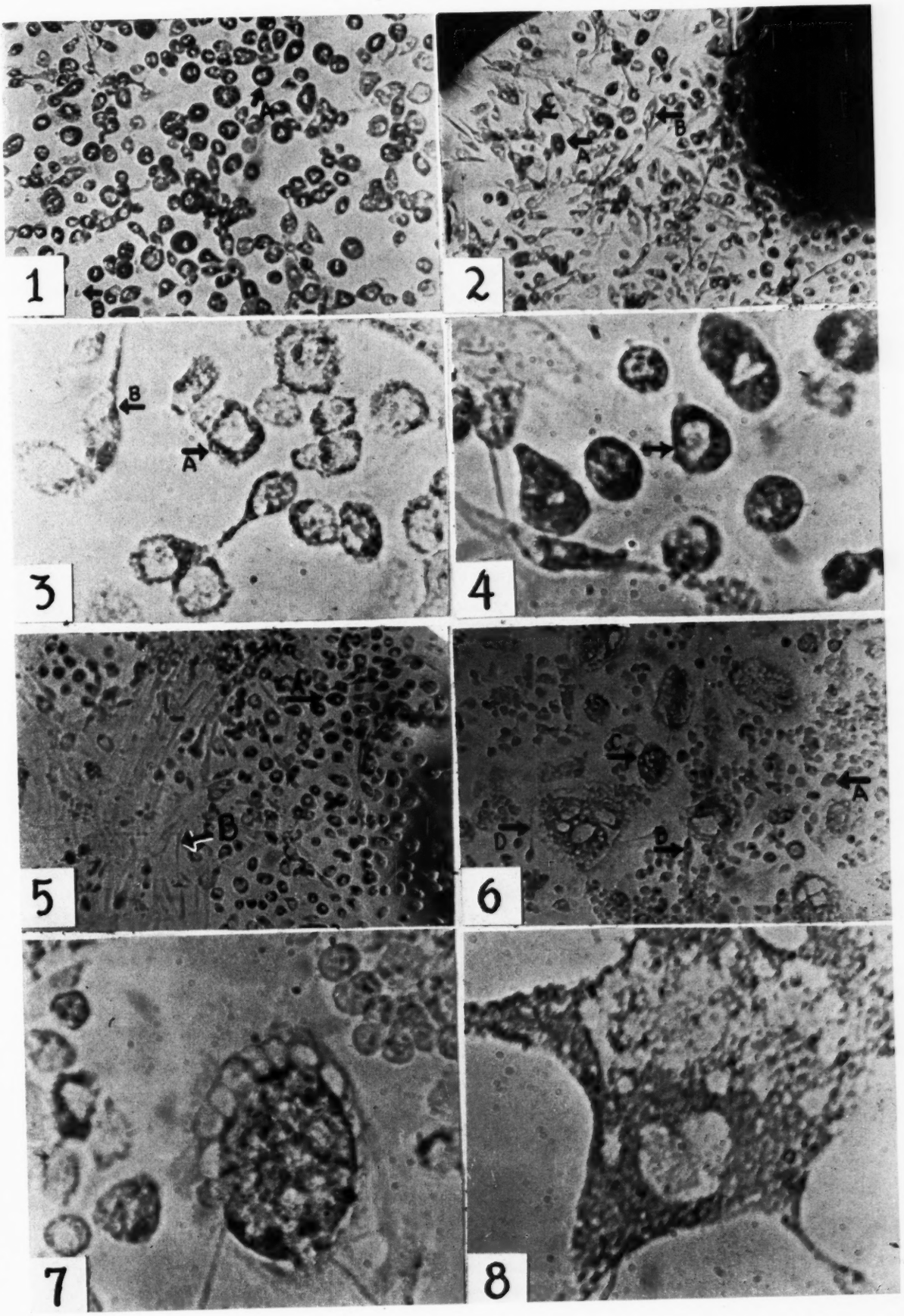
FIG. 4.—Hodgkin's lymph node, Type I. The same type of growth as Figs. 1 and 2 (at higher magnification). The arrow illustrates a macrophage-type cell. 10-day culture. Mag. × 1500.

FIG. 5.—Hodgkin's lymph node, Type I–Ia. Macrophage-type cells containing fat bodies in association with fibroblasts growing in loose irregular network. The cell types indicated by arrows are: (A) the macrophage-type cell and (B) the fibroblast. 14-day culture. Mag. × 240.

FIG. 6.—Hodgkin's lymph node, Type I. Ring form and foreign body giant cells. The cell types indicated by arrows are: (A) the macrophage-type cell, (B) the reticulum cell, (C) the ring form giant cell, and (D) the foreign body giant cell. 6-day culture. Mag. × 300.

FIG. 7.—Hodgkin's lymph node, Type I. Giant cell, ring form type. 6-day culture. Mag. × 1500.

FIG. 8.—Hodgkin's lymph node, Type I. Giant cell, foreign body type. 6-day culture. Mag. × 1500.



FIGS. 1–8

imbedded in a fibrinogen and thrombin coagulum at intervals extending from the lower to the upper end of each tube. One cc. of the following nutrient fluid was then introduced into each tube: 2 parts Gey's solution, 1 part normal placental serum, and 1 part normal adult serum.

The tubes containing explanted lymph node material were stoppered with rubber vaccine or serum bottle tops and were incubated at 37.5° C. in a rotating roller-tube wheel (8 revolutions per hour). Each culture was nourished biweekly with fresh fluid after removal of the fluid in contact with the cells during the previous 3-day period.

Information concerning the following criteria was recorded serially for each culture: (*a*) the relative proportion of fixed cell and free forms; (*b*) the extent and rate of growth; (*c*) the degree of liquefaction; and (*d*) the types and individual characteristics of cells. Data relating to the presence, length of time present, number, type, size, granule or fat body content (number, size, and refractility), and number of nuclei of the following cells was recorded: lymphocytes (Fig. 1), fibroblasts (Figs. 2, 5, 9, and 10), reticulum cells (Figs. 2, 3, and 6), macrophages (Figs. 1–6), and giant cells, "ring form" and "foreign body" type (Figs. 6–8).

Microscopic observations (100× and 500× magnification) were made daily on new and actively growing cultures and biweekly on older and more slowly growing cultures. Photomicrographs of representative, unstained, living explants within the roller tube were taken weekly, or more often, as indicated by the rate of growth of the cultures. Growth in the roller tube was allowed to continue for long periods of time (3–25 weeks) without transplantation to another tube.

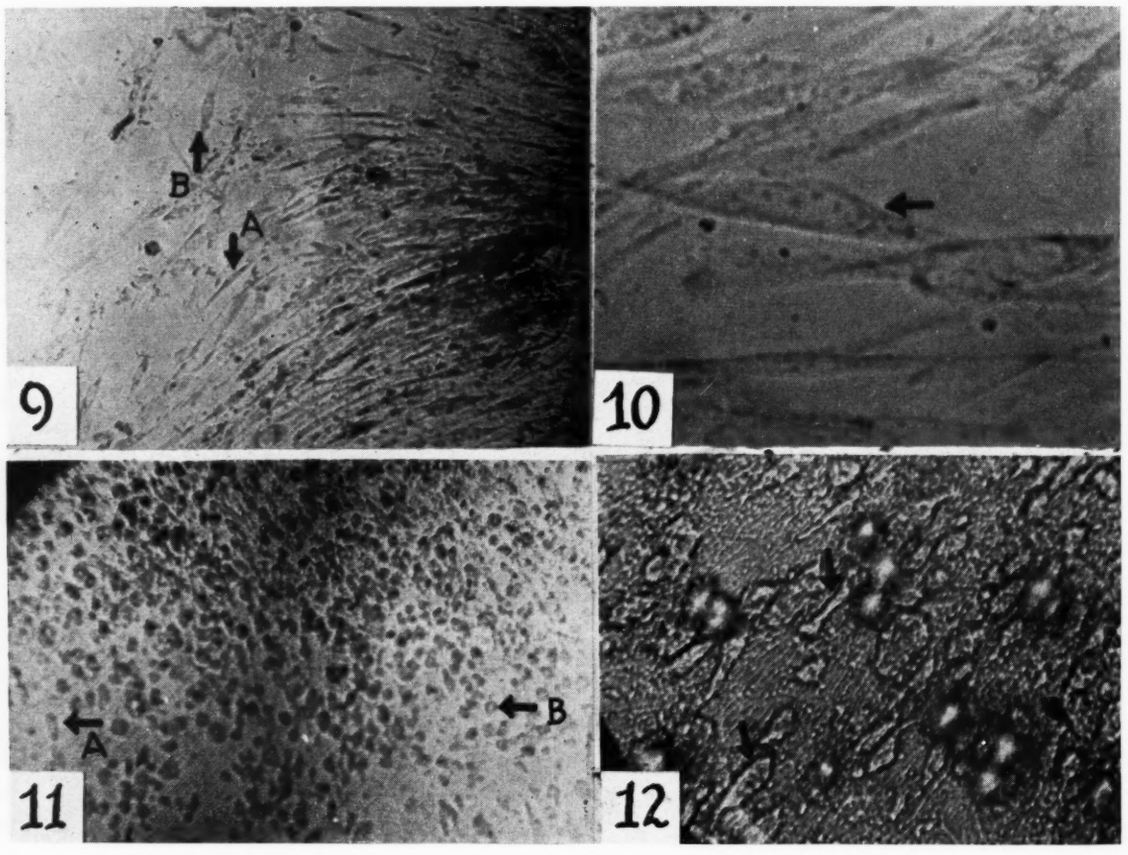


FIG. 9.—Hodgkin's lymph node, Type Ia. Fibroblasts growing in loose irregular network are illustrated. The cell types indicated by arrows are: (A) the fibroblast and (B) the reticulum cell. 17-day culture. Mag. × 240.

FIG. 10.—Hodgkin's lymph node, Type Ia. The same type of growth as Fig. 9 (at higher magnification). The arrow points to a fibroblast. 6-day culture. Mag. × 1500.

FIG. 11.—Hodgkin's lymph node, Type II. Granule-free "abortive fibroblasts" and free cell growth are illustrated. The cell types indicated by arrows are: (A) the "abortive fibroblast" and (B) the macrophage-type cell. 6-day culture. Mag. × 300.

FIG. 12.—Hodgkin's lymph node, Type II. The same type of growth as Fig. 11 (at higher magnification). The arrows point to "abortive fibroblasts." 178-day culture. Mag. × 780.

RESULTS

A tissue culture study of 50 Hodgkin's nodes revealed two growth types. These two types were tentatively classified as the following: (*a*) type I: the granule-containing, free cell, macrophage and reticulum-cell type (Figs. 1–8), (*b*) type Ia: the network or fixed-cell fibroblast type (Figs. 9 and 10), and (*c*) type II: the granule-free, free cell, "abortive fibroblast" type (Figs. 11–13).

Thirty-five (70 per cent) of the lymph nodes cultured were found to be type I (Figs. 1–8), 8 (16 per cent) type Ia (Figs. 9 and 10), and 7 (14 per cent) type II (Figs. 11–13). In eight of 50 (or 16 per cent of the explanted nodes) a combination of types I and Ia was observed in single explants (Fig. 5). Rare, granule-containing macrophages were observed in 1 of 7 type II cultures; a few free-growing fibroblasts were observed in 5 of 7 type II cultures. No "abortive fibroblast" type cells occurred in those cultures classified as types I and Ia. A description of the morphology and growth patterns of types I, Ia, and II follows (Table 1).

Type I.—The granule-containing, free cell, macrophage, and reticulum-cell type is identified by a predominance of reticulum cells and round macrophage-type cells and the presence of a free cell type of growth (Figs. 1–8). The presence of granules (bodies stained with Sudan IV) in large numbers within the macrophage-type cells and reticulum cells is the most prominent feature observed. These bodies range in size from 300 mμ to 1 μ, a value determined from a series of electron micrographs of tissue culture cells representing the explantation of five type I lymph nodes. The following variations within type I were noted: granule-containing macrophages or reticulum cells were the predominant cells or were present in equal numbers. Although most of the reticulum cells had a few short pseudopodia (Fig. 2), a few greatly extended cells containing a few to moderate numbers of granules were also observed, as were a few to moderate numbers of fibroblasts in loose, irregu-

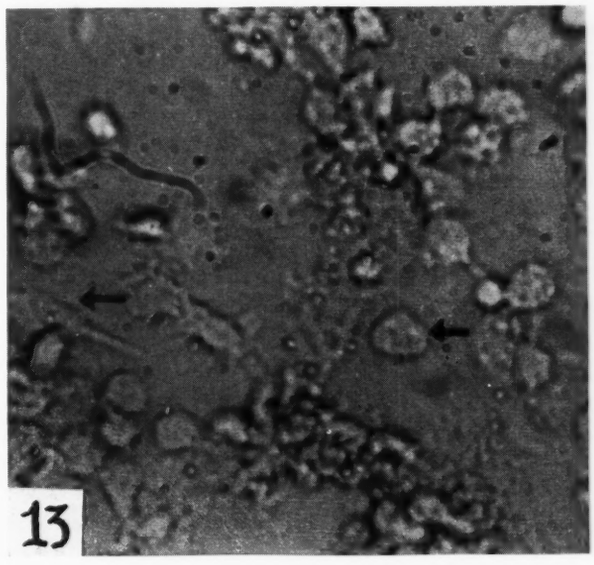


FIG. 13.—Hodgkin's lymph node, Type II. The same type of growth as Fig. 11 (at higher magnification). The arrows point to "abortive fibroblasts." 2-day culture. Mag. × 1500.

TABLE 1

A COMPARISON OF HODGKIN'S LYMPH NODE TYPES I, Ia, AND II IN TISSUE CULTURE*

DESCRIPTION OF CULTURE	TYPE I Granule-containing, free cell, macrophage and reticulum cell type	TYPE Ia Network or fixed cell fibroblast type	TYPE II Granule-free, free cell, "abortive fibroblast" type
Free forms	++++	+	++++
Network (fixed cell) forms	0 to ++++	++++	0
Lymphocytes, number	++	++	++ to ++++
Reticulum cells, number	++++	++	0
granule content	++++	+	0
"greatly extended"	+	0	+
Macrophage-type cells, number	++++	+	++
granule content	++++	+	0†
"Abortive fibroblasts," number	0	0	++++
granule content	0	0	0
Fibroblasts, number	+ to ++++	++++	+
granule content	+	+	0
Giant cells, number	+ to +++	+	+
nuclei, number	2–12	2–4	2–15
Liquefaction, degree	0 to ++++	++	+ to +++
Growth, extent of	+ to ++++	+ to +++	+ to +++
Length of life	6 weeks	5 weeks	6 weeks

* 0 = none or absent; + = few or rare; ++ = moderate number; +++ = many; and ++++ = predominant cell form.

† Rare, granule-containing macrophages were observed in one of seven type II cultures.

lar network (Fig. 2)—a small number of these containing granules. Cultures of 8 of the 35 lymph nodes classified as type I contained many granule-free fibroblasts in loose irregular network (Fig. 5) and were considered a combination of types I and Ia. A few to many giant cells (Figs. 6–8) were present, contained two to twelve nuclei, and were irregularly shaped or oval with nuclei scattered throughout the cytoplasm, or oval with nuclei arranged in a ring at the periphery of the cell. Many giant cells contained a few to moderate numbers of granules. Although a moderate number of lymphocytes (Fig. 1) were present initially, a wide variation in the number of lymphocytes remaining during the life of the cultures was noted. Liquefaction of the clot ranged from 0 to 4+ at the end of the growth period. Outgrowth of cells from type I cultures ranged from fair to excellent, and the average length of life of the cultures (without transplantation) was 6 weeks.

Type Ia.—The network or fixed cell fibroblast type, unlike most type I and all of type II cultures, contains many fixed cell forms which appear to be fibroblasts growing in loose, irregular patterns (Figs. 9 and 10). These fibroblasts have long processes and are in most cases granule-free or contain a few granules. A moderate number of reticulum cells, some of which contain a few granules and approximately one-half of which occur in the free state, were usually associated with these cultures (Fig. 9). A moderate number of lymphocytes were present initially for a short period of time. None of the "abortive fibroblast" forms observed in type II were observed in these cultures. Granule-containing, round, macrophage-type cells were rarely observed. Giant cells with two to four nuclei were occasionally observed. The degree of liquefaction at the end of the growth period was recorded as 2+ and is considered to be somewhat less than that observed in types I and II. The growth of cells observed in type Ia cultures ranged from fair to good, and the average length of life of the cultures (without transplantation) was 5 weeks.

Type II.—The granule-free, free cell, "abortive fibroblast" type is characterized by a free cell form of growth and by the presence of a cell form which appears to be an "abortive fibroblast" incapable of further differentiation (Figs. 11–13). This cell is rounded or elongated, and, although it appears to be a fibroblast, it is not attached to or connected with other cells and lacks definite pseudopodial processes in most cases. Bodies stained with Sudan IV (fat granules) are absent in this cell form, and the cell types observed in I and Ia cultures are not morphologically comparable to it. A moderate number to many lymphocytes were observed throughout the life of these cultures. A moderate number of round, granule-free, macrophage-type cells were usually present (Fig. 11), and rare macrophage-type cells containing a few granules were observed in one of seven examples of this type cultured. A few granule-free fibroblasts growing in the free state and a few greatly extended granule-free reticulum cells were also present. Giant cells with two to fifteen nuclei of the ring form and foreign body types were occasionally associated with type II cultures. These giant cells contained few granules. Liquefaction at the end of the growth period was recorded as 1+ to 3+. Outgrowth of cells from type II cultures ranged from fair to good, and the average length of life of the cultures (without transplantation) was 6 weeks (the maximum was 25 weeks).

A study, based on the criteria of Jackson and Parker (4), of the fixed and stained sections cut from tissues, a part of which were explanted in tissue culture, failed to reveal consistently demonstrable differences among tissues which had been classified by tissue culture as types I, Ia, and II. A tabulation of more than 50 categories of observation involving clinical and laboratory findings (exclusive of the biopsy findings mentioned above) suggested the following minor differences in patients previously classified by tissue culture as type I, Ia, and II. Patients classified as type II, unlike types I and Ia, appeared to have softer, more freely movable and more discrete lymphadenopathy. Clinical observations in this case are limited to five patients classified as type II on the basis of seven cultured biopsies.

Biopsies were repeated on three patients classified as type I at $2\frac{1}{2}$, 6, and 12 months, and two as type II, at 8 and 18 months, following the first biopsy; the type of *in vitro* growth observed originally was obtained a second time in each of the five patients studied.

No correlation was established between the stage of the disease observed in the patient and the histology of the explant growth. Most of the nodes cultured were obtained from patients in the early or intermediate stage of the disease. Fibrous, hard lymph nodes in most cases exhibited poor growth or did not grow in tissue culture.

DISCUSSION

The presence of more than one type of explant cell (types I and II) may furnish evidence favoring the further subdivision of Hodgkin's disease. For this reason, clarification of the significance of these different cell types may be of potential sig-

nificance and practical value. The present observations fail to confirm, except in part, the often repeated premise that the histology of the explant growth reflects the "stage of the disease" at the time of biopsy. The authors have not observed, for example, that a fibrous node is always associated with advanced disease or that the type and degree of reticulum cell abnormality is associated with the stage of the disease.

Morphologic observations in Hodgkin's explant growth suggest that there is a deficiency in the formation of fixed cell or network forms, since 84 per cent of the explants (types I and II) contain a predominantly free cell type of growth. The development of fat bodies within the macrophage and reticulum cells in Hodgkin's disease explants is abnormally great (type I), possibly because of a deficiency in lipolytic enzymes or the presence of an inhibitor. The presence of the so-called "abortive fibroblast" forms (type II) in certain explants suggests an additional functional impairment of the reticulo-endothelial system.

The failure of cell types observed in type II explant outgrowths, and in most cases in type I and Ia, to change morphologically during the course of growth extending over periods from 3 to 25 weeks suggests that these cells, like other neoplastic cells, may have lost the power of differentiation. Transplantation of cultures and the use of growth-stimulating embryo extracts were avoided in an effort to promote and preserve the morphologic evidences of function often necessary to the visual differentiation of one cell type from another (1).

When one considers the tissue culture findings of other workers with respect to the rate of growth and the predominance of fibroblastic growth in Hodgkin's disease, it is difficult to escape the conclusion that the transplantation of cells and the nutrient material used in the culture are important, both in relation to the resulting so-called overgrowth of the entire culture with fibroblasts and the degree of cellular differentiation. Observations in this laboratory suggest that maintenance of the original culture without transplantation and the use of a bovine-human tissue culture system, containing no chicken embryo constituents, appear to insure growth while eliminating the "frequent overgrowth of fibroblasts" observed by many workers. The fibroblasts which are observed in type Ia grow in an irregular loose pattern, unlike the growth of normal fibroblasts, and do not appear to grow luxuriantly or to overgrow the other elements of the culture.

Attempts to repeat, by serial biopsies and explantation of cells, the findings observed initially in the present study have been made in a small number of cases; although the results are encouraging in indicating that the same cell type returns in tissue culture on a second biopsy, they do not prove that more than one histologic type of Hodgkin's disease exists because of their small number. Preliminary observations concerning clinical differences among patients whose type has been differentiated by tissue culture must be extended numerically in order to justify the suggestion that such differences exist.

Because of the nature of the morphologic differences between types I, Ia, and II in tissue culture (i.e., the presence or absence of fat bodies, cohesion of cells, and fixed cell growth), it was expected that the attempted demonstration of comparable differences in the formalin-fixed, hematoxylin- and eosin-stained biopsy section would be difficult. For this reason, the failure to establish a correlation between tissue culture types and fixed tissue biopsy sections was not considered to deny the potential significance of the *in vitro* observations described above.

SUMMARY

1. A tissue culture study and classification of 50 Hodgkin's lymph nodes at biopsy has resulted in the designation of the following two histologic groups:

Type I: the granule-containing, free cell, macrophage and reticulum-cell type, Ia, the network or fixed cell fibroblast type, and I–Ia—a combination of I and Ia.

Type II: the granule-free, free cell, "abortive fibroblast" type.

2. An unsuccessful attempt to correlate differences in tissue culture types with differences in fixed tissue biopsy sections is described. Further observations are needed to confirm or deny the existence of certain minor clinical differences among patients classified by tissue culture as type I, Ia, and type II.

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The Changes in the Mitotic Mechanism of Human Cancer Cells

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Many of the mitotic irregularities characteristic of cancer cells had already been described by the end of the last century. Good reviews of the older literature on this subject are found, for instance, in Politzer (18) and Caspersson and Santesson (2). Our knowledge of these phenomena has, however, greatly increased during recent years. This is to a large extent due to the development of new staining methods and of the squash-technic in making preparations, which have brought the human chromosomes within the reach of much more exact observation than was possible earlier.

The most important work on chromosomal conditions in human cancer cells has been carried out by Koller (11–13), who has described and explained a great variety of mitotic abnormalities in them. Our observations agree in their main features with those made by Koller. Now, however, such a wealth of data on this subject has accumulated that the next important task seems to be the establishment of a common ground for their interpretation. The present observations seem also to have an important bearing on the much discussed question of the origin of cancer.

MATERIALS AND METHODS

The present study is based on 174 cases of carcinoma of the female genital tract diagnosed and/or treated in the Women's Clinic (I and III) of the University of Helsinki during a period from the end of 1948 to the end of 1949. This material falls into the following groups: vaginal carcinoma, 4 cases; cervical carcinoma, 92 cases; corpus carcinoma, 53 cases; ovarian carcinoma, 24 cases; and one case of carcinoma of the fallopian tubes. All the biopsies were taken from untreated cases. The chromosomes of normal tissue were examined from 35 cases of endometrium in the proliferation stage. Since earlier observations, as well as our own, show that, as regards the chromosomal conditions, the different types of carcinoma agree in all their pertinent features, we have in this connection considered the different cases together.

The chromosomes in normal and cancer tissue were studied both from squash and sectioned preparations. The squashes were made according to the technic described in 5, 7, and 10. The material was fixed in acetic-alcohol (1:3) for 1 hour. The Feulgen method was mainly used for staining, i.e., a hydrolysis of 8 minutes in 60° C. N HCl; this was followed by staining in Feulgen's leuco-basic fuchsin for 2–3 hours. Thereafter, the biopsies were squashed and the preparations made permanent (5). This method gave the best results with human chromosomes and was, therefore, mainly used. Some minor modifications were, however, tried. Thus, a slight staining in acetic-lacmoid after Feulgen often improved the staining of the chromosomes. Light green was also applied as a counterstain for Feulgen with good results (7).

To obtain a histological picture of the cases in question, paraffin sections (4 and 10 μ) were examined. The material for these was fixed in 10 per cent formol and stained with Feulgen, crystal violet, or van Gieson's triple stain (19). With the two latter methods, the spindle structure, which is completely invisible in squash preparations, is beautifully revealed.

The figures have been drawn with the aid of camera lucida with a ×25 ocular and ×90 objective, and the final magnitude is ca. ×2000. The photographs have been taken with a Zeiss camera using a ×18 ocular and ×90 oil immersion objective, the final magnification being ×1500.

The diagram in Figure 1 is based on a count of 200 dividing cells in each case. The shading illustrates the percentage of irregular divisions in which chromosomes were seen lagging outside the plates, irrespective of whether the cells were diploid or polyploid.

OBSERVATIONS

Chromosomes in normal cells.—To obtain a basis for the interpretation of the cytological phenomena characteristic of cancer, material from normal endometrium in the proliferation stage was examined for comparison. The most obvious feature of the normal chromosomes, as compared with

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those in cancer cells, was their much better fixability. The stickiness so often described in cancer chromosomes is absent, and the chromosomes fix and stain excellently. Even with the rather crude method of fixation in 10 per cent formol combined with van Gieson's staining, this difference is discernible. The divisions appear regular as a rule; no lagging chromosomes are visible either at metaphase or anaphase.

A normal metaphase plate containing 48 chromosomes is seen in Figure 2, illustrating the different chromosome types described in man (15). In accordance with earlier observations, we expected

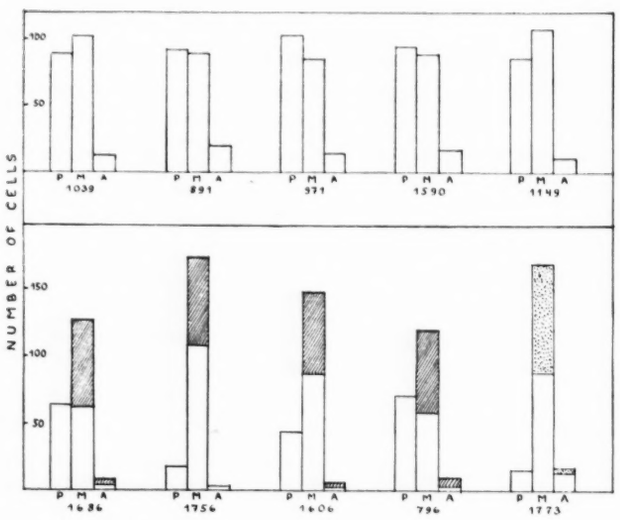


FIG. 1.—The relative frequencies of prophase (P), metaphase (M), and anaphase (A), stages as counted from 200 cells. The upper row: cases of normal endometrium; the lower row: cases of carcinoma. Shading indicates irregular divisions, stippling multipolar divisions.

this chromosome number to prevail exclusively in the normal somatic cells of man. Our expectations were, however, not fulfilled. In a great number of endometrium cells the chromosome number was considerably lower than 48. In Figure 3, an example of a prometaphase stage with 18 chromosomes is seen. In addition to hypoploid cells, polyploid cells, which have presumably arisen through endomitosis, may also be observed in the normal endometrium.

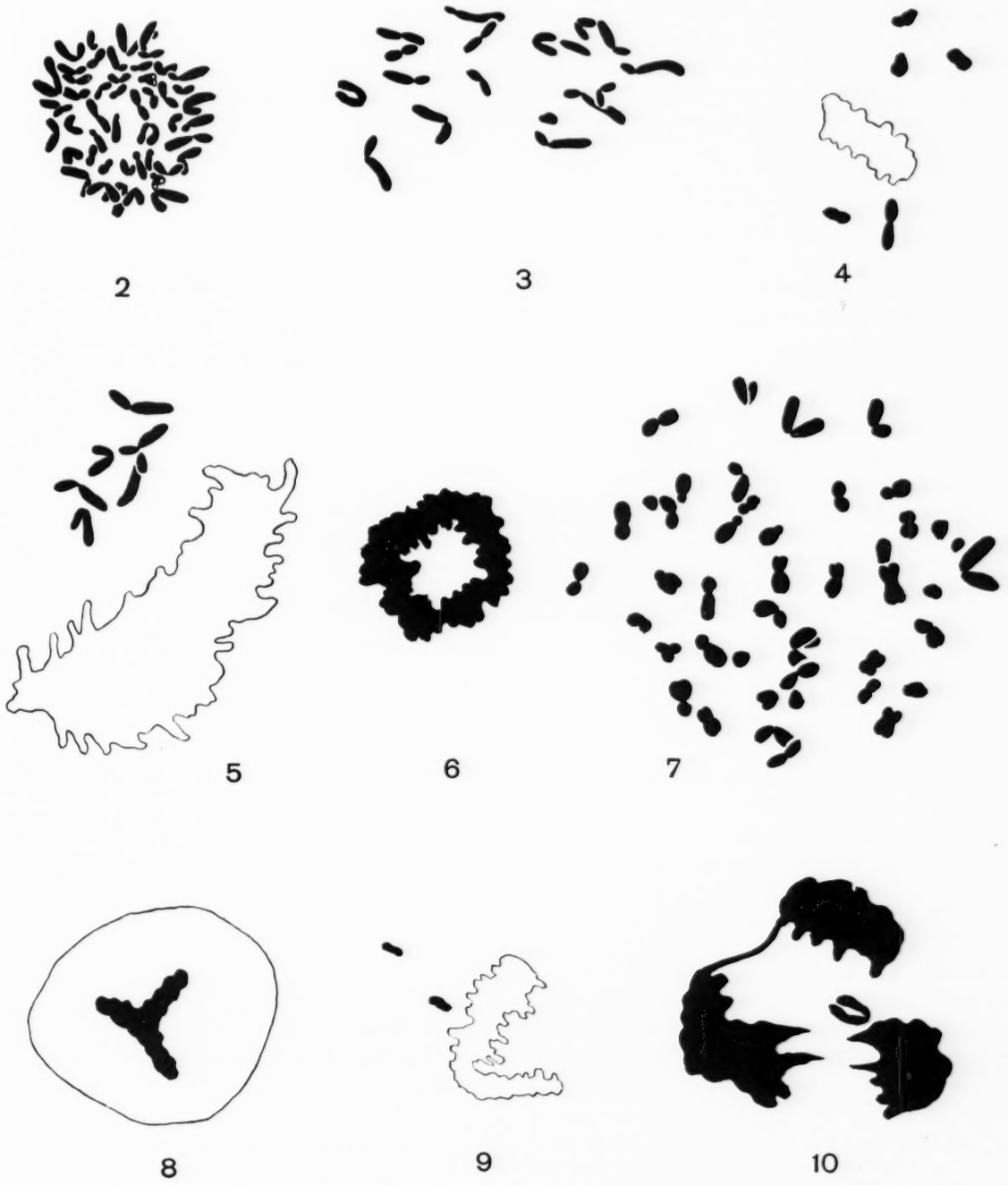
It has usually been thought that chromosome numbers deviating from the normal are not found in somatic cells of an individual; and Koller (12)—to take one example out of many—especially stresses that aneuploid cells characterize malignant tissues. It may, however, be pointed out that grave doubts concerning the presumed constancy of the chromosome number in somatic cells of a given organism have been raised lately (cf., especially, Huskins [8]).

The present finding will be considered in more detail in another paper (Timonen and Therman, unpublished). In this connection it seems worth pointing out, however, what interesting implications this phenomenon has for cytology as well as ontogeny.

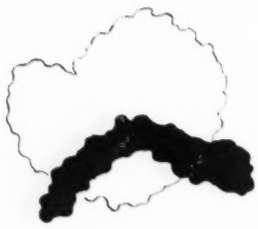
Cytology of cancer cells.—A characteristic feature of neoplastic tissue is that many more cells are seen dividing, as a rule, than in the corresponding normal tissue. In the normal endometrium in the proliferation stage, when the division rate is at its height, the frequency of mitosis is, however, about the same as in cancer tissue. Whether the frequency of divisions in cancer tissue depends on a greater number of dividing cells or on a change in the length of the mitotic cycle, or on both, could naturally be decided only from an examination of living cells.

A fact which is evident from fixed material is that the relative duration of the different mitotic stages in cancer cells is considerably changed. In Figure 1 we see the relative frequencies of prophase, metaphase, and anaphase stages, as determined from five cases of normal endometrium and five cases of cancer. In each case, 200 dividing cells have been counted. In the normal endometrium, the number of prophases slightly exceeds that of the metaphases, as a rule. In the neoplastic tissues, the number of prophases is greatly reduced relative to the metaphase stages. The relative number of anaphase stages shows also a tendency to decrease. Case 1149, in which the number was somewhat higher than the number of prophases, suffered from metropathia haemorrhagica. On the basis of the present observations, the ratio of prophases/metaphases would seem to be a fairly reliable indicator of malignancy—which might possibly even have practical importance.

The relative frequencies of the mitotic stages may be interpreted as indicating their relative duration. The relative duration of prophase stages is thus greatly reduced in cancer cells (cf. 13), and it seems fairly safe to conclude that their absolute length is also shortened. This would mean that the formation of the spindle is more rapid in cancer cells. Other facts are in good agreement with this assumption. In many cases half the metaphases displayed lagging chromosomes outside the plate (Figs. 4, 5, 23). The frequency of divisions exhibiting this kind of irregularity are indicated by the shaded columns in Figure 1. In case 1773, the divisions display so many kinds of irregularity that the metaphases and anaphases containing lagging chromosomes cannot be scored. Instead, the stippled parts of the columns show the frequency of multipolar divisions which constitute about one-half of the total.



FIGS. 2–3.—Cells of normal endometrium.
FIGS. 4–10, cancer cells.
FIG. 2.—Normal metaphase stage with 48 chromosomes.
FIG. 3.—Hypoploid cell with 18 chromosomes.
FIG. 4.—Metaphase with lagging chromosomes.
FIG. 5.—Polyploid metaphase with lagging chromosomes.
FIG. 6.—Hollow metaphase plate.
FIG. 7.—A cell with 43 chromosomes showing the "colchicine-effect."
FIG. 8.—Small tripolar metaphase.
FIG. 9.—Tripolar metaphase.
FIG. 10.—Tripolar anaphase displaying stickiness.
(FIGS. 8 and 10, Fo, F; the others, AA, F; AA = acetic alcohol; Fo = formol; F = Feulgen.)



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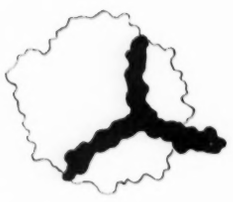

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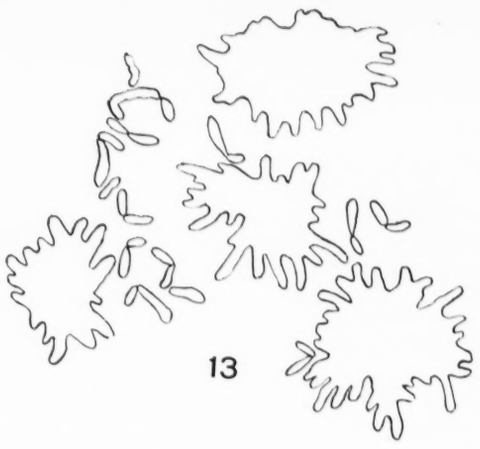

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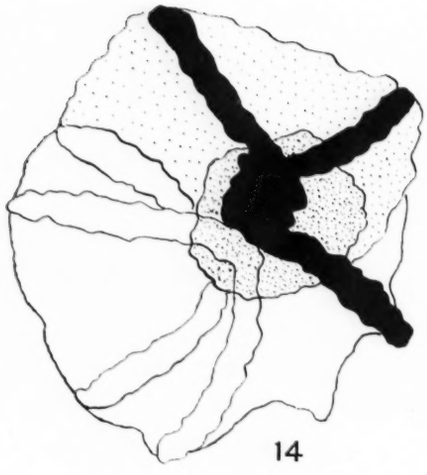

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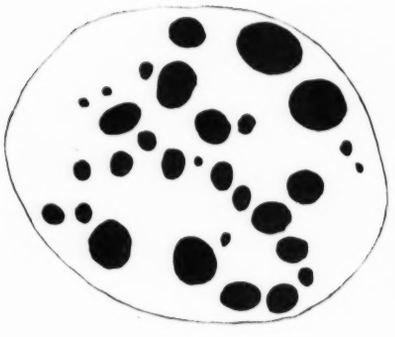

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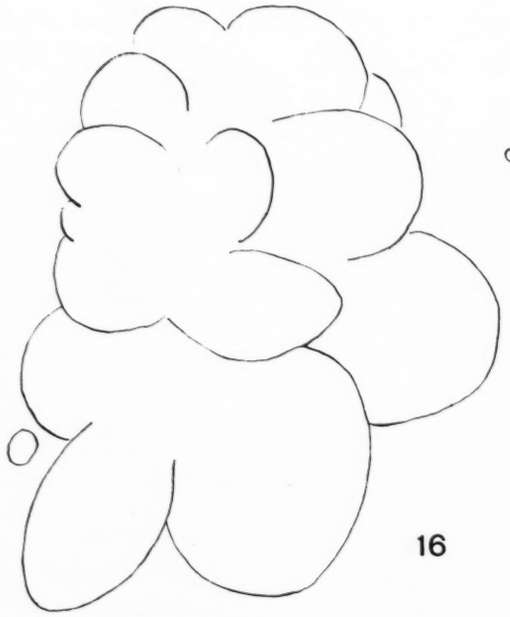

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FIGS. 11–17.—Cancer cells.

FIG. 11.—Pentapolar metaphase.

FIG. 12.—Quadripolar metaphase.

FIG. 13.—Quadripolar anaphase with lagging chromosomes.

FIG. 14.—Highly multipolar metaphase.

FIG. 15.—Degenerating nucleus, the chromatin in droplets.

FIG. 16.—Many-lobed giant nucleus.

FIG. 17.—Octopolar anaphase with lagging chromosomes.

(FIGS. 11–13 AA, F; the others, Fo, F.)

In anaphase, the laggard chromosomes (Fig. 21) are either included in the separating chromosome groups or divide afterward. Even when no true laggards are seen outside the metaphase plates, the chromosomes show a much less strict orientation than in normal cells. This is especially clear in the polyploid plates in which the chromosomes often lie in several layers.

The failing orientation of the chromosomes naturally gives rise to the extremely variable chromosome numbers found in tumor cells (18, 12). The same variation in the chromosome number has been observed by us (e.g., Fig. 18). As described above, the lowered aneuploid chromosome numbers are not, however, restricted to malignant cells. The same is true of polyploid cells, except perhaps the very high polyploids. In Figure 24, a giant prophase stage is illustrated; it seems as if the chromosomes were in part in a state of fragmentation.

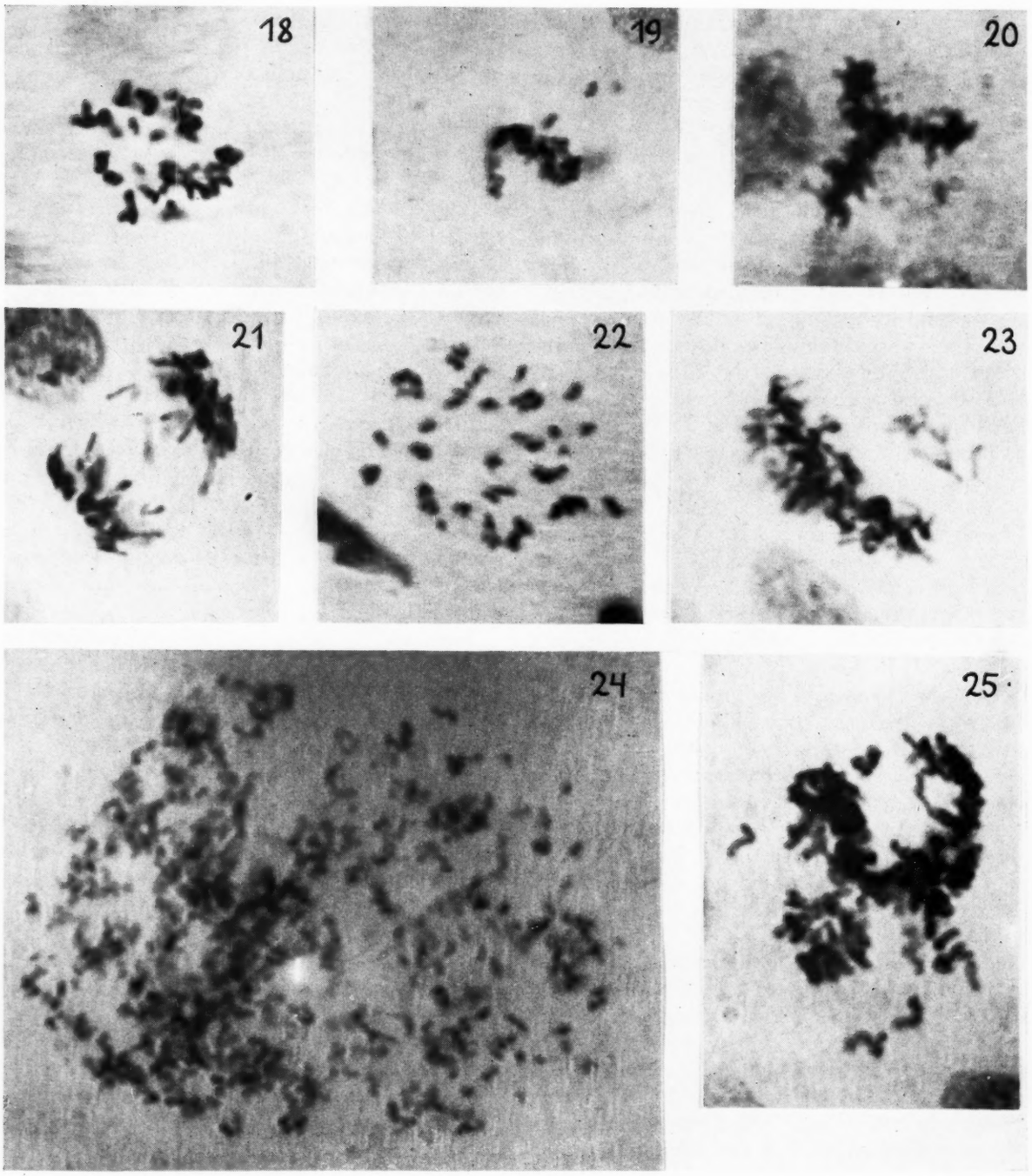
A further feature observed in the mitoses of cancer tissue is the occurrence of hollow metaphase plates (Figs. 6, 25), which are absent in the normal endometrium. We have observed all intermediate configurations between normal metaphase plates and completely hollow plates in which the center is without chromosomes. The hollow ring is often broken, also. The degree of hollowness is correlated neither with a small chromosome number nor with greater stickiness of the chromosomes. It may tentatively be suggested that even this phenomenon is ultimately caused by the rapidity of the spindle mechanism. Hollow spindles have been observed in man in the spermatogonial mitoses (9) and in the human pre-myelocytes as a result of pernicious anaemia (14).

The stickiness of the chromosomes in cancer cells has often been described. As stressed by Koller (12, 13), different tumors, and even neighboring cells in the same tumor, vary greatly in this respect. The stickiness of the chromosomes has been attributed to an excess of deficiently polymerized nucleic acid (11). As seen in the figures, the same variation in the stickiness of the chromosomes is evident in our material, too. It seems to us that no decisive role can be ascribed to the stickiness of the chromosomes—although often present in cancer cells—in the origin of cancer, since it is often absent and the chromosomes in certain cases may even display the contrary appearance of undercharging with thymonucleic acid. In this connection, the studies of Biesele (1) on the chromosomes in the lymphocytes of leukemic mice may be mentioned. These chromosomes, which showed an increased size as compared with normal lymphocyte chromosomes, shrank with pepsin digestion more than normal chromosomes. This indicates, in Biesele's opinion, that the greater size of the former depends on an increase of nucleic acid as well as of pepsin-digestible protein.

A number of cancer cells exhibit features which closely resemble the effects induced by colchicine and other drugs. The chromosomes lie scattered around the cell, being at the same time more contracted than usual. In their division, they show failing synchronization. It is seen in many cells that a number of chromosomes have already divided, others are just dividing, forming typical "c-pairs," while the rest show no signs of division (Figs. 7, 22). In these cells the spindle mechanism has evidently broken down, and the failing coordination leads to defective synchronization. Some of the neighboring cells in a tissue may show this "colchicine-effect," while others appear unaffected. It might be thought that this phenomenon is a result of the abnormal metabolism in neoplastic tissues (6). The same may be the cause of the fragmentation of the chromosomes in cancer cells (12).

Favorable material for the investigation of multipolar mitoses was provided by a case of ovarian carcinoma (1773) in which one-half of all the mitoses seemed to be multipolar. In this carcinoma, all cell types from actively dividing diploid or hypoploid cells to giant multipolar cells evidently containing up to one thousand chromosomes were encountered. Although the giant cells often had several poles, multipolarity by no means always coincides with polyploidy. We have found very small tripolar metaphases (Fig. 8) as well as polyploid plates which are regularly bipolar (Figs. 5, 23). Regarding Figure 8, it may be mentioned that this cell was situated in a tissue strand which consisted entirely of very small, evidently hypoploid cells. This shows, in our opinion, that Koller's idea (12, 13) that the hypoploid cells in cancer tissue are able to live only when supported by cells with higher chromosome number cannot be accepted as such.

The most usual type of multipolar divisions are tripolar divisions; examples are seen in Figures 8 and 20. A modification of this basic shape is seen in Figures 9 and 19. In this case, chromosome plates have been formed only between two of the three poles. Quadripolar metaphases are illustrated in Figures 12 and 25, while Figure 11 provides an example of a pentapolar metaphase. In Figure 14 a highly multipolar metaphase stage is depicted. It is, however, too complicated to be analyzed in detail. In Figure 10 we see a tripolar anaphase in which the chromosomes are very



FIGS. 18–25.—Microphotos of cancer cells.

FIG. 18.—Hypoploid cell with 32 chromosomes.

FIG. 19.—The same as Fig. 9.

FIG. 20.—Tripolar metaphase.

FIG. 21.—Bipolar anaphase with laggards.

FIG. 22.—Cancer cell showing the "colchicine-effect"; some chromosomes are divided, others not.

FIG. 23.—The same as Fig. 5.

FIG. 24.—Giant prophase stage.

FIG. 25.—Quadripolar metaphase, at the left a similar tripolar configuration to that in Fig. 20, the fourth plate at the upper right hand corner is hollow. (AA, F.)

sticky. Between the separating chromosome groups, three chromatid bridges have been formed, of which two have broken and one has persisted. Figure 13 again provides an example of a quadripolar anaphase, in which the chromosomes are slender and well fixed. Figure 17 again illustrates an octopolar anaphase. All the multipolar divisions, at least in anaphase, contain lagging chromosomes which evidently will fail to be included in the daughter nuclei. It may be mentioned, in passing, that Koller (12, Fig. 8) has depicted a stage which he interprets as a multinucleate cell, in which the chromosomes have formed metaphase plates synchronously. In our opinion, this figure represents a multipolar anaphase analogous to our Figure 17. In our material (especially case 1773) which is rich in multipolar configurations, the course of the divisions could well be followed. In addition, the synchronously dividing multinucleate cells—which are also found in our material—have thicker chromosomes, clearly differing from the more slender anaphasic chromosomes.

The multipolar anaphases form either several nuclei, in which case a multinucleate cell is often formed, or a many-lobed restitution nucleus (Fig. 16). It seems, however, that when the cell has reached a certain size it becomes inviable. In the giant multipolar metaphases it is often seen how the chromosomes begin to disintegrate, forming amorphous chromosome masses. A final stage in this series is seen when the chromatin collects into droplets (Fig. 15). The same phenomenon is reflected by Figure 1 (case 1773), which shows that the percentage of multipolar metaphases in the total is considerably higher than the percentage of multipolar anaphases. This suggests that a number of multipolar metaphases never reach the anaphase stage.

DISCUSSION

The precocity of the spindle mechanism.—The normal mitosis consists of two main processes: the reproduction and division of the chromosomes, and the functioning of the spindle mechanism. These may also be called the intrachromosomal changes as opposed to the extrachromosomal changes in the cell. In normal mitosis, these processes act in step with each other. The reproduction of the chromosomes in prophase (in this connection the much discussed problem of the actual time of reproduction of chromosomes is irrelevant) is regularly followed by spindle formation and the congression of the chromosomes in the metaphase plate, where the "double" chromosomes divide.

Various deviations from the normal course of mitosis have shown that the spindle mechanism and the intrachromosomal processes are to a great extent independent of each other (3, 20). Of the regulated processes in which the spindle cycle and the chromosomal cycle are out of step, the most important is naturally meiosis. Regarding this interpretation of the meiotic phenomena, we might cite Darlington (3, p. 48): "This difference can be expressed by saying that in meiosis the changes outside the chromosomes are advanced in relation to the changes inside the chromosomes. In meiosis, as compared with mitosis, the external changes are precocious." This precocity theory of meiosis has been further developed by Oksala (16) in his study concerning the premeiotic spermatogonial divisions in the dragonflies of the genus *Aeschna*. He comes to the important conclusions that precocity is a phenomenon which develops slowly during the last premeiotic divisions and that it implies more the premature beginning of the polarized stage in the cell, i.e., metaphase, than the premature beginning of prophase, as the precocity theory so often has been interpreted.

As pointed out by Oksala (16), not only meiosis, but a great variety of other cytological phenomena are explicable on the basis of changed timing relations between the intrachromosomal and extrachromosomal processes. We have, thus, all intermediates between endomitosis, which implies the division of the chromosomes without any division of the cell, and somatic reduction, in which the cells divide without any division of the chromosomes (8).

In our opinion, the cytological phenomena characteristic of cancer cells would best be explained along the same lines. Cancer tissue, as is well known, is characterized by an increased rate of cell division. This increased rate seems especially to be brought about by an acceleration in the cycle of the spindle. This, again, is shown by the greatly reduced duration of the prophase stage. The extrachromosomal processes in the cell are, however, not only absolutely accelerated. They also seem to be more rapid as compared with the intrachromosomal changes. In other words, as in meiosis, the spindle mechanism in cancer cells is precocious as compared with the chromosomal mechanism.

Naturally, the regularity governing the meiotic phenomena is absent in cancer, but the same tendency toward a precocity of the extrachromosomal changes is clearly noticeable. The spindle mechanism needs to be, in the beginning, only a little in advance of the chromosomal processes. This desynchronization grows, however, in the following divisions, leading finally to the gross abnormalities observed in cancer cells.

The precocity of the spindle mechanism is reflected also by the behavior of the chromosomes.

In most cases, a considerable proportion of the metaphase stages exhibits lagging chromosomes outside the plate. Obviously, the spindle has been too rapid for them. This happens in polyploid as well as hypoploid cells. The tendency of the metaphase plate to become more or less hollow may also be the result of the acceleration of the spindle.

The multipolarity of the divisions might be interpreted in the same terms. The occurrence of the very small tripolar and quadripolar divisions shows that these by no means always need to be the result of the formation of restitution nuclei. A small quadripolar division would arise when the precocity of the spindle mechanism has reached such a degree that the centrosomes divide twice while the chromosomes divide only once, and the occurrence of higher multipolars could be explained similarly.

As pointed out above, the irregular desynchronization of the different processes in the division of cancer cells leads to numerous cytological abnormalities. Thus, not even the centromeres divide simultaneously, and this results in the formation of the commonly observed tripolar divisions as well as the higher multipolar cells with an uneven number of poles. Comparable phenomena have been observed by Peters (17) in the cornea of Triturus during recovery from colchicine treatment. He ascribes the occurrence of multiple star-like configurations (p. 48) as "due to an abnormal increase in the number of centrioles or to a lack of coordination between the chromosomal cycle and the division of the centrioles."

It was noticed quite early that certain cytological features in cancer cells resembled meiosis (11, 18). In the light of the present observations, this resemblance need not be so superficial as has been supposed (12). The common features might be brought about by the precocity of the spindle mechanism shared by cancer cells. The stickiness of the chromosomes in cancer cells might be caused by the same factors which give rise to the somewhat similar appearance of the meiotic chromosomes. The behavior of lagging chromosomes at metaphase and anaphase exhibits features similar to univalent chromosomes in meiosis.

It cannot be denied that a number of the cytological phenomena observed in cancer are seemingly in contradiction to the precocity of the spindle postulated by us. Thus, for instance, the formation of polyploid cells through endomitosis is a process pointing in the opposite direction. It must, however, be remembered that polyploidy due to endomitosis is not uncommonly found in normal tissues of various organisms. Certain other phenomena, e.g., the "colchicine-effect" and the fragmentation of chromosomes, we ascribe to secondary changes in the tumor tissue due to abnormal metabolism.

Theories concerning the origin of cancer.—The various theories concerning the origin of cancer agree in that they all postulate a somatic mutation in the widest sense of the word. Such a mutation might be: (*a*) genic, depending on a gene mutation; (*b*) chromosomal, being the result of a change in the number or structure of the chromosomes; (*c*) heterochromatic, being brought about by a change in the amount of heterochromatin; or (*d*) plasmatic, implying that the change has taken place in the so-called plasmagenes. According to the first three hypotheses, the change which leads to the development of cancer takes place in the chromosomes, whereas according to the fourth it is to be sought in the cytoplasm.

The chromosomal-mutation theories of the origin of cancer may be traced back as far as Boveri (references to the older literature in 18 and 21), who assumed that the varying chromosome numbers observed in cancer cells were the cause of their malignancy. A serious objection may be raised to all the theories involving the chromosomes that, since actively dividing cancer cells display chromosome numbers ranging from 12 to very high polyploids, a chromosomal change can hardly be thought to be the ultimate cause of their malignancy (4). This argument gains further important support from our present observation that the chromosome numbers also vary greatly in cells of normal tissue.

According to the more recent hypothesis advanced by Caspersson and Santesson (2), the origin of cancer is to be sought in a change of the heterochromatic portions of the chromosomes. This hypothesis is based on spectrographic determinations of the various cell components which have shown that malignant cells contain more nucleic acids than normal cells, and that the rate of protein formation is also increased in them. Since, according to Caspersson and his associates, the nucleic acid metabolism and protein formation in the cell are governed by the heterochromatic portions of the chromosomes, Caspersson and Santesson have come to the above conclusion. This hypothesis is, however, open to severe criticism. The whole concept of heterochromatin seems to be so diffuse and poorly defined, being used by different authors in different senses, that such a hypothesis cannot be based on it. Especially as regards the role of heterochromatin in human cells, it is cytologically as well as physiologically all but unknown. The most important reason for rejecting this hypothesis is, however, the same as that presented above

in regard to the other theories of the origin of cancer based on chromosomal changes.

If we consider the cytological observations made on cancer cells, we find that the most constant feature characterizing all the cases studied is the general acceleration in the division rate. This is connected with the accelerated rate of the spindle mechanism. The other cytological peculiarities seem to be more or less directly caused by this primary change. All these data best agree with the plasmagene theory of the origin of cancer proposed by Darlington (4). According to this theory, the origin of cancer is to be sought in a mutation of the plasmagenes. This would bring it into relation with genes, plasmagenes, proviruses, and viruses, which form an integrated system from heritable particles, the genes, to viruses capable of infection. It is noteworthy how well our observations fit in with Darlington's conclusions arrived at from quite a different angle. Indeed, we might say that our data furnish the necessary illustration and proof for the following assumption presented by Darlington on theoretical grounds (4, p. 124): "In cancer I am supposing a cytoplasmic change which favours a high growth rate. In these circumstances both the nucleus and certain cytoplasmic constituents might well be unable to stand the pace."

SUMMARY

1. The chromosomes in 35 cases of normal endometrium in the proliferation stage have been studied. In addition to the normal chromosome number, 48, both polyploid and hypoploid cells have been found.

2. The chromosome conditions in 174 cases of carcinoma of the female genital tract have been examined. Here also, cells from low hypoploids to very high polyploids have been observed. Other phenomena characterizing cancer cells are the occurrence of lagging chromosomes in the divisions, multipolar spindles, stickiness of the chromosomes, and the "colchicine-effect."

3. The most constant features noticed in cancer cells are, however, the increased division rate of the cells and the changed relative duration of the different mitotic phases, as determined from their relative frequencies. In normal cells the prophase is usually a somewhat longer stage than the metaphase. In cancer tissue, it is much shortened—the metaphase, in this case, being of considerably longer duration.

4. On the above facts we present the following theory: The primary cytological change in cancer cells consists in an accelerated rate of the spindle mechanism. The intrachromosomal changes do not stand the pace, and thus the extrachromosomal changes become precocious in relation to them. From this original change, most of the other chromosomal aberrations can be derived.

5. Of the various theories concerning the origin of cancer our observations agree best with Darlington's plasmagene theory.

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The Virus-induced Rabbit Papilloma-to-Carcinoma Sequence II. Carcinomas in the Natural Host, the Cottontail Rabbit*

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The virus found by Shope (14) in cutaneous papillomas from Western cottontail rabbits, genus Sylvilagus, has been shown to be readily transmissible to both domestic and wild rabbits[1] with the production of vigorously growing tumors (4, 9, 10, 14, 19). The growth pattern of the tumor in both hosts is essentially similar (19). In the first paper of the present series (19), we emphasized that this growth pattern in its final, or involutionary, phase can terminate many months after induction in sharply different forms, the manifestations of which are either regression and disappearance of the lesion or replacement of the papillomatous by cancerous tissue.

In earlier studies (5, 13) and reviews (6–8), it has been reported that malignant change in the natural host, the cottontail rabbit, is a rarity but that cancer in the common domestic rabbit is to be expected. Much has been made of the theoretical importance of this supposed difference (6–8). Since it seemed improbable to us that the cottontail rabbit, a host species shown to be susceptible to carcinogenic agents (11, 12, 17) should be refractory to the development of cutaneous cancer, a colony of cottontails was kept by us for observation (19), to learn the incidence of malignant change and to provide carcinomatous tissue for other experimental studies (20). The development of cancers in 33 of 167 cottontail rabbits kept under observation made it fully apparent that our earlier discovery of cancer in a cottontail rabbit (16) did not represent a rarity.

It is the purpose of the present paper, the second in the series, to report the results of our studies which have established a diagnosis of cancer in 33 of 167 cottontail rabbits, kept under observation without manipulative interference with the natural papilloma-to-carcinoma sequence. Anatomical changes and recovery of virus as related to the cancers and to other growth phases, are also described.

A study of previous reports (5, 13, 16) reveals why cancer in cottontail rabbits following naturally occurring or experimentally induced papillomatosis has come to be accepted as a rarity. The pertinent information is presented in Table 1.

It will be seen in Table 1 that nine cottontails with cancer have been recorded. All but one of these rabbits were infected under natural conditions. The period of observation was limited to a few months. Three of the animals were subjected to biopsies and to autotransplantation of those tissues. The single experimentally infected rabbit had been under observation for nearly 2 years when a cancer (at but one of two sites) was discovered at necropsy. Adequate evaluation of a possible relationship of papilloma virus to this single cancer is hardly possible, because each of this rabbit's experimentally induced papillomas had received three injections of Scharlach R in olive oil and had been subjected several times to operative interference for examination at biopsy. When the cottontail died months later, spindle-cell sarcomas were present at both sites, with metastatic spread to a regional lymph node, and the concomitant epidermoid cancer was found at only a single site.

MATERIALS AND METHODS

The constitution and management of our rabbit colony and the general methods of study have been detailed in the first paper of the present series (19).

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[1] The species of wild hares of proved susceptibility to infection by Shope's papilloma virus include the wild cottontail rabbit (*Sylvilagus floridanus alacer* Bangs (2, 3, 14), *Sylvilagus floridanus malluris* Thomas (14), and *Sylvilagus floridanus mearnsi* Allen (10, 18)); the jack rabbit (*Lepus californicus* Gray (10) and *Lepus californicus melanotis* Mearns (6)); and the snowshoe rabbit, *Lepus americanus* Erxleben (2, 5, 6).

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The present report describes the sequence of papilloma-to-carcinoma changes observed in the cottontail rabbit. During the period of investigation, the population of the rabbit colony was replenished regularly so as to have in stock at all times approximately 150 rabbits. From June, 1934, to April, 1944, 301 tumor-bearing cottontails came under observation. Since, with a single exception, cancer was not diagnosed until the animals had been in captivity for 6 months or longer, 6 months was taken as an arbitrary point for analysis for the allocation of alterative changes. Any rabbit that had survived captivity or utilization in other experimental studies for 6 months, accordingly, was set aside until it died from intercurrent disease, cancer, or accidental trauma. At autopsy, to which each animal was subjected, each tumor was sectioned for histologic study. The resultant histologic diagnosis provided accurate information for this report.

RESULTS

The cottontail rabbits that were kept to permit observation of the natural succession of alterative changes in the papilloma-to-carcinoma sequence fall into two groups: cottontails that were infected in their natural habitat in Kansas, and cottontails that were experimentally infected under laboratory conditions. Because most of the factors known to influence infection by the papilloma virus—dosage, infectious titer, preparation and size of skin area, etc.—were controlled for the cottontail rabbits that were experimentally infected and for the domestic rabbits utilized in comparative studies, the information that relates to experimental infection of the natural host is presented first (Table 2).

It can be seen in Table 2 that the alterative changes in experimentally induced papillomas on 94 cottontail rabbits were assessed in their relationship to the age of the tumor. Regression and disappearance, which represent the ultimate in the involutionary phase of the tumor, were noted for two of thirty rabbits as early as the first 3 months, but in no instance after the sixth month. In contrast, the lesions that underwent malignant change had been present for at least 12 months on six rabbits. Since histologically benign lesions were not found after the fifteenth month, the results raise for consideration the possibility that all papillomas surviving early regressive forces ultimately become cancerous.

TABLE 1

SUMMARY OF INFORMATION THAT RELATES TO PAPILLOMA-TO-CARCINOMA SEQUENCE FOR NINE COTTONTAIL RABBITS

Case No.	Environment Under Which Infection Was Induced	Period Of Observation (days)	Interference with Natural Sequence	Status of lesions at time of death: Benign	Cancer (grade) 1°	Cancer (grade) 2°	Regression	Identification Used In Report	Year Reported	Ref.
1	Natural	91	None	3	1	+	0	S5-CR 363	1935	(16)
2	Laboratory	683	3 injections of Scharlach R in olive oil beneath lesion; several biopsies		1*			W.R.-26	1936	(13)
3	Natural	63	Biopsies and auto-transplantations	Several	9	+	0	W.R. 1-55		
4	"	37	"	0	4	0	0	W.R. 1-56		(5)
5	"	7	"	0	3	0	0	W.R. 1-99		
6	"	2	None	0	1	+	0	W.R. 1-53	1940	
7	"	0	"	0	1	+	0	W.R. 1-54		
8	"	"Few weeks"	"	1	1	0	?	W.R. 66		
9	"	"Several months"	"	0	1	0	0	W.R. 1-39		

* Death after 683 days. Spindle-cell sarcoma at both primary sites with 2° to node.

Table 3 presents the information relating time, as reflected by the period of observation, to the growth phase of the papillomas that had been contracted by 207 cottontail rabbits in their natural habitat.

It can be seen from Table 3 that the period of time during which regression occurred extends from less than 3 to 27 months. Malignancy, too, became apparent within 3 months and thereafter occurred comparatively regularly over a period of 33 months. The incidence of benign lesions fell progressively, but many tumors persisted for more than a year. The lesions that are included on this group were present when the rabbits first came under observation. Obviously, therefore, the time in months presented as the age of the tumor actually represents the period of time during which the animals were under observation. The range of time during which benignancy, malignancy, and regression were observed, therefore, is greater for

this group than for the group of rabbits that had been infected under laboratory conditions.

Table 4 summarizes the information relating to the lesions on the 33 cottontail rabbits with histologically proved cancers.

Thirteen of these 33 rabbits were known to have had 29 papillomas that underwent involution and disappeared. When the 33 rabbits underwent autopsy, 17 carried 59 benign papillomas. Virus was recovered from 21 of the 40 papillomas tested. In contrast, none of 106 carcinomas on removal from dictable and separable into three phases: (*a*) the *proliferative phase* with maximal response of all infected cells resulting in a rapidly growing epithelial tumor; (*b*) the *stationary phase* characterized for prolonged periods of time by no change in the appearance of the tumor; and (*c*) the *involutionary phase* made apparent either by progressive desiccation and regression of the tumor, resulting in its disappearance within weeks, or by proliferation and radical alteration in cellular constituents to result in malignancy, metastases, and death.

TABLE 2

INCIDENCE OF ALTERATIVE CHANGES IN RELATION TO AGE OF TUMOR IN EXPERIMENTALLY INFECTED COTTONTAILS

Age of tumor (months)	Cottontail Rabbits			Growth Phase					
				Benign		Malignant		Regression	
	Eastern	Western	Total	(number)	(per cent)	(number)	(per cent)	(number)	(per cent)
0–3	8	22	30	28	93	0		2	7
3–6	21	18	39	37	95	"		"	5
6–9	5	5	10	10	100	"		0	
9–12	2	4	6	6	"	"		"	
12–15	2	3	5	3	60	2	40	"	
15–18	1	1	2	0		"	100	"	
21–24	"	0	1	"		1	"	"	
24–27	"	1	1	"		"	"	"	

TABLE 3

THE INCIDENCE OF ALTERATIVE CHANGES IN RELATION TO THE PERIOD OF OBSERVATION AMONG NATURALLY INFECTED COTTONTAILS

Period of observation (months)	Number of Western cottontail rabbits	Growth phase					
		Benign		Malignant		Regression	
		(number)	(per cent)	(number)	(per cent)	(number)	(per cent)
0–3	44	37	84	1	2	6	13
3–6	51	"	73	0		14	27
6–9	31	15	48	4	13	12	39
9–12	34	17	50	5	15	"	35
12–15	20	6	30	4	20	10	50
15–18	12	5	42	"	33	3	25
18–21	7	2	28	3	43	2	28
21–24	3	0		"	100	0	
24–27	"	1	33	1	33	1	33
30–33	2	0		2	100	0	

26 rabbits yielded virus. The eventual fate of the papillomas on a single rabbit included as many as five variations: papillomas that regressed, papillomas that yielded virus, papillomas that failed to yield virus, papillomas that became primary cancers, and cancers that metastasized to lymph nodes and lungs. We regard this finding as most important.

DISCUSSION

The data presented in the present paper and in the first paper of the series (19) show that the growth pattern of the tumor initiated by rabbit papilloma virus (Shope) is not measurably altered by the variety of rabbit host employed. After a variable incubation period of from 7 to 42 days, the virus, irrespective of the host species, initiated a papilloma with a growth cycle that was pre-

The findings summarized above emphasize the complete similarity in the succession of changes that occur in the evolution of the papilloma-to-carcinoma sequence in both varieties of rabbit, cottontail and domestic. Yet, despite the similarity in cellular response, important differences in host reactivity early became apparent, as evidenced by ready recoverability of virus from cottontail rabbit tumors, whereas virus was not recoverable, or but rarely recoverable, from the papillomas of domestic rabbits. This difference in host reactivity was especially striking in the cottontail, for the proliferative phase of growth in this host not uncommonly persisted to yield virus for many months until the onset of the involutionary phase, but virus disappeared with the first evidence of regression or alteration in cellular constituents to malignancy. Other evidence of host differences was

the observation that the gross removal of papillomatous tissue from cottontail rabbits, whether by inadvertent trauma or by Roentgen radiation, was followed by the renewal of growth with replacement of the papillomatous tissue, whereas such growth was not resumed after total removal of papillomas from domestic rabbits.

The similarities and the differences in host response must be kept in mind, since the literature revealed that cottontails had been seldom used for the investigation of the papilloma-to-carcinoma sequence. This failure to study the disease in the natural host undoubtedly resulted because of the many difficulties attending the capture, husbandry

TABLE 4

SUMMARY OF DATA RELATING TO 33 COTTONTAIL RABBITS WITH EPIDERMOID CARCINOMA PROVED AT AUTOPSY

Distribution of lesions on 33 rabbits with proved cancer		Total number according to: Diagnoses by: Rabbit host	Diagnoses by: Number of lesions	Recovery of virus by: Rabbit host	Recovery of virus by: Number of lesions
Benign papillomas		17	59	5/26*	12/40
Involution of benign papillomas with disappearance		13	29		
Epidermoid carcinomas	Primary cancer	33	69	0/26	0/106
	Metastases	19	26		

* Denominator: number tested. Numerator: number positive.

and handling, the maintenance of an adequate supply, and a shorter survival period when in captivity. Moreover, the domestic rabbit, the use of which is free from such difficulties, has proved readily susceptible to infection by the virus. Quite naturally, therefore, there has been a large experience with the papilloma-to-carcinoma sequence in domestic rabbits and but a limited experience with the phenomenon in cottontail rabbits. In our opinion this situation has led to unwarranted conclusions.

With the growth of our appreciation of the fact that cancer occurs frequently in cottontail rabbits, we restudied previously reported cases. That cancer was proved for eight cottontails with naturally occurring papillomas and for one with experimentally induced papillomas is set forth in Table 1. Case 1 showed that the papilloma-to-carcinoma sequence can occur in the absence of purposeful interference in the natural host of the virus, the cottontail rabbit, and that an epidermoid carcinoma, metastatic carcinoma, and benign papillomas can co-exist in a single animal. The other seven cottontails, which had been infected under natural conditions (Cases 3–9), provided adequate demonstration of the occurrence of carcinomatous degeneration in naturally occurring papillomas. It may be this small group of rabbits, selected from several thousand and sent by a Kansan trapper to Kidd and Rous in response to their request for rabbits with abnormal lesions, that led these workers (5) to conclude that seven among several thousand rabbits represents the natural incidence of cancer. Further evidence, to them, that the papilloma-to-carcinoma sequence in cottontail rabbits was rare was their finding (Case 2) of an epidermoid carcinoma 683 days after infection with papilloma virus. In this instance, moreover, the natural sequence of events was obscured by early and repeated infiltration of Scharlach R in oil under the papillomatous lesion and by the demonstration at autopsy of spindle-cell sarcomatous tissue at the sites of injection and in metastases. The other eight cases yielded no information as to the length of time required for the papilloma-to-carcinoma sequence to occur, since advanced alterative changes were present when the animals first came under observation.

SUMMARY

Cancers commonly develop from virus-induced papillomas (Shope) in the natural host, the cottontail rabbit. Thirty-two of the 127 cottontail rabbits which were kept under observation for more than 6 months yielded 106 tumors, which were proved to be epidermoid carcinomas by histologic study. Metastases occurred in 19 of the 32 rabbits. Attempts to recover papilloma virus from the carcinomas met with failure in 106 instances; yet the virus was readily recovered from 12 of the 40 benign papillomas which were removed from 26 of the 32 rabbits that also had proved cancers.

The development of cancers from papillomas in more than one-fourth of the cottontails under observation is similar to the development of cancer in the experimental host, the domestic rabbit. It may be concluded from these results that many of the hypothetical considerations which were founded on the supposed rarity of cancer in the natural host for the papilloma virus, the cottontail rabbit, are no longer tenable.

The observations of differences in host reactivity, as described in this paper, were accepted as especially worthy of investigation (20), since, if virus is present in the ultimate cancer, it should be more readily demonstrable with the cottontail rabbit as the experimental host.

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Serum Polysaccharide Levels in Rats Bearing the Walker 256 Tumor*

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Previous work on human subjects (9) has shown that the polysaccharide associated with serum proteins is significantly elevated in malignancy. Little is known concerning the correlation between this elevation and the cancerous condition. Work in this laboratory has shown that the serum polysaccharide of dogs is increased in several types of experimental inflammation (7). An elevation also occurs in pregnancy (4, 10), tuberculosis, sarcoidosis, and other pathological conditions (6, 9). This communication deals with a study of serum polysaccharide during the progressive development of a rapidly growing transplantable rat tumor.

EXPERIMENTAL

Experimental animals.—Transplants of the Walker 256 carcinosarcoma[1] were made subdermally on either the abdomen or the back, by means of a No. 15 hypodermic needle. Male rats of the Sprague-Dawley strain were used throughout. Age of the rats varied between 65 and 200 days of age at the time of transplantation. A successful transplantation rate of about 95 per cent was obtained. All analyses were made on pooled serum obtained by cardiac puncture from six rats in each group. A set of control animals was placed under the same environmental conditions and bled at the same time as each set of tumor animals. To provide some estimate of the rate of growth of the tumors, the width and breadth of the tumor were measured in millimeters each week. These measurements were then multiplied together; the results of this calculation are shown graphically in Figures 1 and 2.

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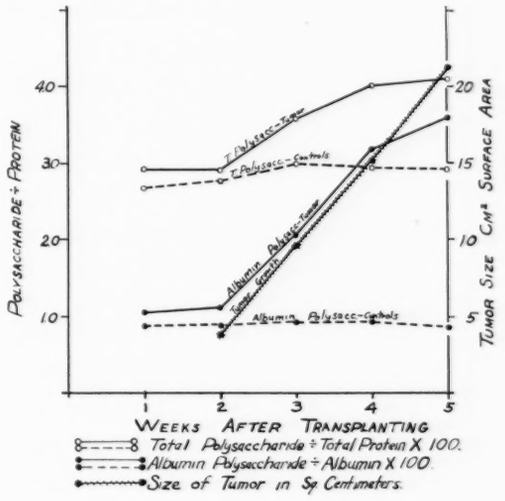

Fig. 1.—Changes in polysaccharide content of the total serum and of the albumin fraction. Solid lines denote averages of tumor groups. Dotted lines denote averages of control groups.

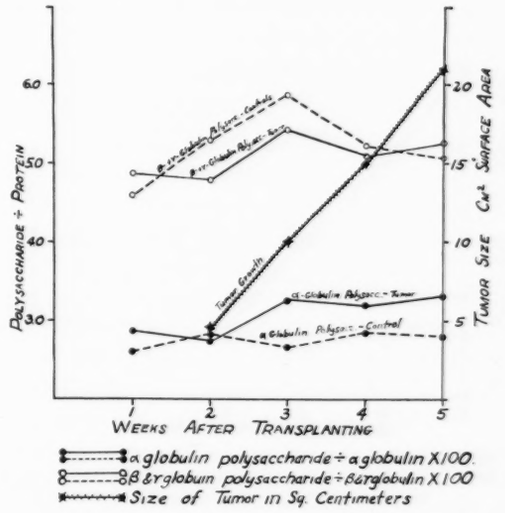

Fig. 2.—Changes in polysaccharide content of the α-globulin and β- + γ-globulin fractions. Solid lines denote averages of the tumor groups. Dotted lines denote averages of control groups.

Chemical methods.—Serum was fractionated into albumin, albumin + α-globulin, and β- + γ-globulin fractions by the method of Milne (3) developed for human plasma, using 19.6 per cent and 26.0 per cent sodium sulfate to precipitate the globulin fractions. Nonglucosamine polysaccharide

TABLE 1

AVERAGE POLYSACCHARIDE CONTENT OF THE SERUM PROTEIN FRACTIONS IN TUMOR-BEARING RATS

	Weeks after transplanting					
	0	1	2	3	4	5
Total nonglucosamine polysaccharide (mg. per cent)						
Tumor animals		169	168	192	186	193
Controls	155	152	162	172	172	173
Albumin polysaccharide (mg. per cent)						
Tumor animals		20	19	32	37	45
Controls	17	18	18	17	17	17
α-globulin polysaccharide (mg. per cent)						
Tumor animals		54	47	47	44	48
Controls	52	45	49	50	55	56

(referred to hereafter in this paper simply as polysaccharide) was determined as previously described (8) by the tryptophane method on the albumin and albumin + α-globulin fractions after ethanolic precipitation (2 ml. of filtrate was added dropwise to 15 ml. of absolute ethanol). The polysaccharide associated with the α-globulin fraction was obtained by difference between the two determinations and the polysaccharide associated with the combined β- and γ-globulin by subtracting the polysaccharide of the albumin and α-globulin fractions from the total serum polysaccharide. Protein was determined on all fractions by the biuret reaction.

RESULTS

Changes of protein distribution.—The average results of the polysaccharide and protein determinations are summarized in Tables 1 and 2. The results shown in Table 2 for normal rats agree fairly well with the electrophoretic data of Gjessing and Chanutin (1), who report 42–45 per cent albumin and 32–34 per cent α-globulin in plasma protein from normal male rats of the Wistar stock. A decrease in serum albumin from 35 per cent to 26 per cent of the total serum proteins occurred in tumor-bearing rats after 5 weeks of tumor growth. Conversely, the β- + γ-globulin fraction increased from 32 per cent to 41 per cent, while α-globulin exhibited no change. During this period the control rats exhibited a small decrease in serum albumin and a slight increase in α-globulin. In this connection it might be noted that Werner (11) noted a similar decrease in albumin and elevation of α-globulin in rabbit sera following massive daily bleedings.

Some of the increase in total serum polysaccharide in the tumor-bearing rats is due to an increase of globulins relative to albumin. Thus, if all serum protein fractions of the tumor-bearing rats had the same polysaccharide content as those of the control rats, the total polysaccharide: total protein ratio 5 weeks after transplantation would be 3.38. The difference between this figure and the actual ratio (4.34) represents a change in the polysaccharide content of the serum fractions.

Changes of polysaccharide distribution.—The serum or serum fraction levels expressed by a figure obtained by dividing the polysaccharide level by the amount of protein in a given fraction are depicted graphically in Figures 1 and 2. These data were obtained by averaging results from four different groups of rats: two with transplants to the abdomen, one with transplants to the back, and one with transplants to both abdomen and back. No difference was noted among the groups. In a similar way, data for the controls were obtained by averaging results for the control groups carried simultaneously with each group of tumor-bearing animals.

The total nonglucosamine polysaccharide of the serum increased noticeably by the third week in all tumor-bearing groups. The protein polysaccharide of the albumin fraction was elevated noticeably after 2 weeks of tumor growth and became markedly elevated as the size of the tumor increased. This was found to be true for each group of rats as well as for the average of all groups. It is noteworthy that the increase in albumin polysaccharide roughly paralleled the figures obtained for the size of the tumor. In general, the polysaccharide content of the α-globulin of the tumor-bearing rats was slightly elevated after the third

TABLE 2

AVERAGE SERUM PROTEIN DISTRIBUTION IN TUMOR-BEARING RATS

	Weeks after transplanting				
	1	2	3	4	5
Total protein (gm. per cent)					
Tumor animals	5.7	5.3	5.0	4.4	4.3
Controls	5.6	5.5	5.4	5.2	5.7
*Albumin**					
Tumor animals	35	30	29	27	26
Controls	35	32	31	31	31
*α-globulin**					
Tumor animals	33	32	30	32	33
Controls	31	30	35	32	34
*β- + γ-globulins**					
Tumor animals	32	38	41	41	41
Controls	34	38	34	37	35

* Expressed as per cent of total protein.

week, in comparison with that of the control group. However, in one group of rats this elevation was not found. When based on the average of all groups, the polysaccharide content of the combined β- and γ-globulin fraction rose in both tumor and control rats, reached a maximum, and then decreased; but this trend was marked in only one set of animals. It appears doubtful whether the polysaccharide content of the globulin fractions is connected in any direct way with the growth of the tumor.

DISCUSSION

The relatively small elevation of total serum polysaccharide in the normal rats during the course of the experiment was partly due to an increase in the serum α-globulin of this group—possibly a response to the injury caused by the periodic bleeding. The chief cause of the elevation of serum polysaccharide in the tumor-bearing rats is attributed to the increased polysaccharide content of the albumin fraction. This finding is in agreement with those of Seibert, Pfaff, and Seibert (5), who were unable to completely account for the high serum polysaccharide content in human carcinoma patients by calculations based on the polysaccharide content of normal serum fractions and the distribution of these fractions in the sera of carcinoma patients, as determined by electrophoretic analyses. Apparently, there is an increase in some carbohydrate-rich fraction of serum which accompanies albumin during removal of the globulins by 26 per cent sodium sulfate. As results obtained by animal experimentation may not be directly applicable to human metabolism, similar fractionation studies are being conducted on sera from cancer patients. Investigations are also under way to determine what part of the polysaccharide associated with the albumin fraction is due to the mucoprotein fraction described by Winzler and Smyth (2, 12).

SUMMARY AND CONCLUSIONS

A study was made of the effect of tumor growth on the serum polysaccharide level of rats bearing the Walker 256 tumor. The total nonglucosamine polysaccharide increased greatly as the tumor size increased. Much of this increase was due to a polysaccharide associated with serum albumin as determined after the precipitation of globulins with 26 per cent sodium sulfate. The elevation occurred concurrently with an actual decrease in the albumin fraction relative to the globulin fraction in the sera of tumor-bearing animals. In the rat the polysaccharide content of the albumin fraction closely paralleled the tumor size, suggesting a possible relationship of this factor to tumor growth.

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The Effect of the Proportion of Dietary Fat on the Rate of Formation of Mammary Carcinoma in Mice*

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Fat-enriched diets enhance the rate of formation of certain types of tumors in the mouse (1, 11, 16, 20); the subject has already been adequately reviewed (14, 18). In various experiments, performed at different times in our laboratory with the spontaneous mammary carcinoma and the induced skin tumor, diets containing approximately 12 per cent fat appeared to augment tumor formation very nearly as much as those containing about 30 per cent. It therefore seemed worthwhile to investigate the quantitative relation between the degree of fat-enrichment of the diet and the formation of tumors.

For the present experiments, the formation of the spontaneous mammary carcinoma of the mouse was investigated, inasmuch as it is more responsive to the action of dietary fat than is the formation of induced skin tumors (16). Two experiments were performed: in one, the diets were compounded of commercial components; in the other, of semi-purified components.

The results of the two experiments were in excellent agreement, and it was found that the rate of tumor formation (as measured both by incidence and by the average time of appearance of the tumors) tends to increase with increasing proportion of dietary fat. The effect was related to the proportion of dietary fat and was not the result of differences in caloric intakes or body weights of the mice.

GENERAL PROCEDURES

The following conditions and procedures were common to both experiments. The mice, virgin females, were of inbred strains born in our laboratory. From weaning until transfer to the experimental rations they were fed Purina laboratory chow checkers *ad libitum*. The several groups of each experiment were composed, so far as possible, by distribution of litter mates, and the mice were housed in groups of five in cages with solid bottoms. The diets were prepared by mixing a 1-week supply of the weighed components with sufficient water to make easily molded mashes which were spread in pans, cut into blocks of appropriate sizes, and stored in a refrigerator at 38° F. The mice were fed daily. Equicaloric amounts of the ration were fed at a level slightly below the known voluntary intake; this insured average equicaloric intakes[1] among the several groups. The mice were weighed and inspected for tumors biweekly. Each animal was examined at post-mortem. Many tumors were examined histologically, including all those that were doubtful and all those not located in the mammae or liver. Records of the individual mice were kept, which yielded, in addition to the principal data, incidental information on longevity, metastases, and multiple mammary tumors.

EXPERIMENTS

Experiment 1.—Five groups were employed, each consisting of 52 virgin C3H females. They were 9–13 weeks old when transferred to their respective experimental rations. The basal portion of the daily diets for each mouse contained 1.0 gm. Purina Fox Chow meal, 0.5 gm. skimmed milk powder, 0.2 gm. casein, and 0.1 gm. brewers yeast; the remainder of the diets consisted of cornstarch and fat in such proportions that the diets of the five groups ranged from 1.6 per cent to 26.0 per cent fat. All five diets provided 11.4 Calories daily. The amounts of protein, salts, and vitamins were the same among the several rations, whereas the proportions of these constituents increased with increasing proportions of dietary fat. The diets fed groups A121, A122, A123, and A124 contained 1.6, 5.7, 12, and 26 per cent fat, respectively, and the

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[1] The caloric values of the rations, and the protein, mineral, fat, and carbohydrate contents were calculated on the basis of data supplied by the manufacturers of the dietary constituents.

diet fed group A125 contained 24 per cent fat and 2 per cent cholesterol. The diets are detailed in Table 1.

Table 2 illustrates the mean body weights of the mice not bearing mammary tumors at several periods up to 1 year of age; after this the number of tumor-free mice decreased rapidly, and the averages were less reliable. From about the 24th week on, the mice of the two groups ingesting the rations containing 26 per cent fat (A124 and A125) weighed consistently less, on the average, than those on the diets containing 2, 6, or 12 per cent fat. At 7 months of age, the mean body lengths (nose to base of tail) were 10.8, 10.6, 10.6, 10.7, and 10.7 cm., respectively, with a mean standard error of 0.12 cm.

The experiment was terminated when the mice were 2 years old. The results are given in Table 3. C3H strain mice fed calorically and nutritionally adequate diets characteristically develop a high incidence of mammary carcinoma; therefore, no considerable difference in the final tumor incidence was observed among the several groups. That increasing the proportion of fat in the diet does enhance the formation of mammary carcinoma is shown, however, in the increasing tumor incidence (when compared at times up to 80 weeks) and in the earlier average appearance of the tumors. The effects were not arithmetically proportional to the fat intake; the augmentation resulting from an increase in dietary fat from 1.6 to 5.7 per cent (A121 compared with A122) was as great as that of an increase from 5.7 to 26 per cent (A122 compared with A124). The two diets containing 26 per cent fat, either without (A124) or with (A125) added cholesterol, enhanced tumor formation to about the same degree.

TABLE 1

DIETS EMPLOYED IN EXPERIMENT 1

	Grams per mouse per day				Per cent composition		
Group	Basal*	Corn-starch	Kre-max†	To-tal§	Fats	Pro-tein	Min-erals
A121	1.8	1.4	0	3.20	1.6	21	3.3
A122	1.8	1.1	0.12	3.02	5.7	22	3.5
A123	1.8	0.7	0.28	2.78	12	24	3.8
A124	1.8	0	0.56	2.36	26	28	4.5
A125	1.8	0	0.56‡	2.36	26‡	28	4.5

* 1.0 gm. Purina Fox Chow, 0.5 gm. skimmed-milk powder, 0.2 gm. casein, 0.1 gm. brewers yeast (Anheuser-Busch, strain K).

† Partially hydrogenated cottonseed-soybean oil (Armour & Co.).

‡ Including 0.05 gm. cholesterol (2.1 per cent of diet).

§ All diets supplied 11.4 Calories daily. Caloric value of cornstarch, 3.6 Calories per gm.; of Kremax, 9.0 Calories per gm.

Among the tumor-bearing mice in groups A121, A122, A123, A124, and A125, multiple mammary carcinomas occurred in 27, 53, 51, 60, and 53 per cent, respectively. Thus, fewer of the mice fed the ration containing 1.6 per cent fat developed more than one mammary tumor than did those receiving higher proportions of dietary fat.

The survival times of the individual mice, subsequent to the development of mammary carcinoma, ranged from 2 to 25 weeks; the average survival time among the several groups varied from 11.1 to 12.7 weeks and was not related to the proportion of dietary fat. The incidences of grossly visible metastases to the lungs varied from 24 to 38 per cent among the several groups, also showing no regular relation to fat intake.

TABLE 2

GROWTH OF C3H FEMALE MICE ON RATIONS CONTAINING DIFFERENT PROPORTIONS OF FAT (EXPERIMENT 1)

		Age of mice (weeks)							
Group	Dietary fat (per cent)	12	16	20	24	28	36	44	52
		Mean body weight (gm.)							
A121	1.6	22	26	28	32	37	36	37	35
A122	5.7	22	27	30	32	38	37	37	38
A123	12.0	23	26	29	32	37	35	37	36
A124	26.0	22	26	28	30	35	33	32	34
A125	26.0*	23	26	28	30	36	33	34	34

* Including 2.1 per cent added cholesterol.

Experiment 2.—Five groups, each consisting of 60 virgin dba females 10–14 weeks of age, were employed. The diet for each mouse included a basal portion consisting of casein, gelatin, salts, yeast extract, synthetic B vitamins, and a supplement containing vitamins A, D, and E. This basal portion was complemented by fat and cornstarch in amounts which provided different proportions of dietary fat for the five experimental groups: 2.0, 4.1, 8.0, 16.0, and 23.7 per cent. The daily ration for each mouse provided 10.9 Calories. The diets are detailed in Table 4. The experiment was terminated when the few surviving mice were 2 years old.

The proportion of dietary fat had little effect on the rate of weight gain or on the final body weight (Table 5). The results on tumor formation (Table 6) are in agreement with those of the previous experiment: Increasing proportions of dietary fat tended to increase the incidence of mammary carcinoma and to shorten the mean age of tumor appearance. The effects of increasing the fat content from 2 to 8 per cent (AP1 compared with AP3) were as great as those of an increase from 8 to 24 per cent (AP3 compared with AP5). There was a negligible effect, if any, attributable to an increase from 16 to 24 per cent (AP4 and AP5). In Table 6, the incidence of mammary tumors is computed on the basis of an adjusted total of mice, which cor-

rects for deaths of nontumor mice during the course of the experiment (6); this was done because 11–16 of the mice in each group died without tumors. These deaths were not associated with the dietary regimen and were due principally to lymphomata and leukemia (5–7 deaths in each group) and a small number of other primary tumors. A few deaths were associated with uterine infections and granulomas.

and 5.57 ± 0.54 for groups AP1 and AP5, respectively. The proportion of dietary fat did not influence the growth rate of the spontaneous mammary carcinomas.

DISCUSSION

The results of the two experiments are in agreement and indicate that the rate of formation of spontaneous mammary carcinoma of the mouse (as measured both by tumor incidence and average time of tumor appearance) tends to increase with increasing proportion of dietary fat. The rate of tumor formation was not arithmetically proportional to the fat intake; rather, the stimulatory effect was as pronounced when the proportion of dietary fat was increased from approximately 2 per cent to 6 or 8 per cent as when it was increased from the latter levels to 24 or 26 per cent. In fact, there was little effect, if any, produced by an increase from 16 to 24 per cent. In agreement with this plateauing of the effect of dietary fat, Boutwell *et al.* (4) found that a diet containing 61 per cent fat stimulated the formation of induced skin tumors to about the same extent as one containing 27 per cent fat.

This type of relation is not uncommon for many

TABLE 3

FORMATION OF MAMMARY CARCINOMA IN C3H MICE INGESTING DIETS CONTAINING DIFFERENT PROPORTIONS OF FAT

Group	Dietary Fat (per cent)	Number* of mice	Age of mice (weeks) Per cent mice with mammary tumors 40	50	60	70	80	90	104†	Age at appearance of tumors (weeks) Range	Mean	50 per cent age§ (weeks)	Number of mice alive and tumor-free at 104 weeks†
A121	1.6	52	17	29	50	62	62	73	81	27–103	58.4±3.1	59	4
A122	5.7	52	17	48	63	73	75	83	87	29–101	53.3±2.7	52	5
A123	12	52	23	50	68	75	81	90	94	24–100	53.1±2.8	50	2
A124	26	52	31	54	65	79	86	90	94	27– 97	51.7±2.5	48	0
A125	26‡	47	30	57	74	78	85	87	89	31– 99	48.4±2.3	45	4

* Number of mice alive when first mammary carcinoma was observed in experiment (effective totals).

† End of experiment.

‡ Including 2.1 per cent added cholesterol.

§ Age at which 50 per cent of the mice had tumors.

TABLE 4

DIETS EMPLOYED IN EXPERIMENT 2

Group	Grams per mouse per day Basal*	Cornstarch	Kremax	Total†	Per cent composition Fat	Protein	Minerals
AP1	0.855	2.09	0.055	3.00	2.0	23.0	3.0
AP2	0.855	1.94	0.115	2.91	4.1	24.0	3.1
AP3	0.855	1.69	0.215	2.76	8.0	25.0	3.3
AP4	0.855	1.24	0.395	2.49	16.0	28.0	3.6
AP5	0.855	0.89	0.535	2.28	23.7	30.0	3.9

* 0.7 gm. vitamin-free casein (Borden Labco); 0.06 gm. gelatin (United Chemical and Organic Products Co., No. 14X); 0.09 gm. Osborne-Mendel salt mixture, Wesson Modification (21); 0.005 gm. cottonseed oil containing 20 U.S.P. units of Vitamin A, 2 U.S.P. units of D, and 0.8 mg. of E (Distillation Products, Inc.). The B vitamins were supplied in 0.03 gm. yeast extract (Anheuser-Busch, No. 3) and the following synthetic vitamins: thiamin chloride, 60 μg.; pyridoxine HCl, 60 μg.; riboflavin, 30 μg.; folic acid, 12 μg.; biotin 0.03 μg.; niacin, 300 μg.; Ca pantothenate, 180 μg.; *p*-aminobenzoic acid, 600 μg.; inositol, 3 mg.; choline chloride, 15 mg.

† All diets supplied 10.9 Calories daily. Caloric value of cornstarch, 3.6 Calories per gram; of Kremax, 9.0 Calories per gram.

The interval from the appearance of a mammary tumor to the death of the animal ranged from 1 to 25 weeks among the individual mice, and averaged from 9.6 to 10.4 weeks among the several groups, there being no association with the level of dietary fat. Of the mammary cancer mice of groups AP1, AP2, AP3, AP4, and AP5, respectively, 17, 33, 26, 38, and 34 per cent had more than one mammary tumor. The incidence of grossly visible metastases to the lungs was not associated with the levels of dietary fat. The growth rates of all tumors in groups AP1 (2 per cent fat) and AP5 (24 per cent fat) were determined (16); the growth indices (average daily increment in the sum of major and minor axes in units of 0.1 mm.) ranged from 1.0 to 13.3 and averaged 5.61 ± 0.45

biologically active substances, such as vitamins and hormones. These produce successively greater effects as the dose is increased from a low to an optimal level; increases beyond this optimal level have no further effect.

There were no significant differences in caloric intake or body weight among the groups of an experiment, and, therefore, the enhancement of tumor formation was not mediated through either of these two factors.

In contrast to the stimulatory effect of high dietary fat on the formation of spontaneous mammary carcinoma was the lack of effect on the growth of the tumors, survival time of the mice following the appearance of the tumors, and the incidence of metastases to the lungs; all these indicate that fat enrichment of the diet has no significant effect upon the growth of spontaneous mammary carcinomas.

The numerous experiments performed by us and others on the effect of high-fat diets on tumor formation have resulted in probings of the mode of action of this experimental procedure. As yet, no single incontrovertible explanation has been offered.

We have suggested (16, 18) that substituting fat for isocaloric amounts of carbohydrate in the diet may affect the genesis of tumors by at least two means: (*a*) a local action (solvent effect), brought about by an increased fat content of the tissue involved, under which conditions the rate of transfer or amount of carcinogen is altered; and (*b*) an independent effect of fat on the developing tumor cell. This hypothesis was offered to explain the augmenting effect of a fat-enriched diet on the formation of spontaneous mammary and induced skin tumors, and the slight retardation of the genesis of carcinogen-induced sarcoma. The lack of any effect of a high-fat diet on the incidence of spontaneous lung adenoma may be due to the fact that the lung is not a fat depot (13) and is therefore unaffected by varying percentages of fat in the diet. The effect of a fat-enriched diet on tumor formation in a given tissue may depend upon the extent to which the amount of fat in that tissue is modified by the diet.

Recently, Boutwell, Brush, and Rusch (4) have suggested that the increasing efficiency of utilization of diets due to increasing proportions of fat may be the factor responsible for the action of fat-enriched diets in accelerating the formation of skin tumors induced by 3,4-benzpyrene. Although they discussed certain qualifications, they concluded that "the difference in the net energy value of low and high fat diets is sufficient to explain the stimulating effect of fat on the induction of skin tumors with carcinogenic hydrocarbons." This interpretation was based principally on the work of Forbes, Swift, and co-workers (2, 3, 7, 8, 9, 10) who have shown that, in rats, high-fat diets are metabolized with lower net energy expenditure (higher net body energy gain) than corresponding equicaloric low-fat diets. The question arises as to whether the latters' findings may be interpreted as indicating that the enhancing effect of high-fat diets on the formation of tumors occurs through a decrease in energy expense of utilization. The higher net body energy might be considered equiv-

TABLE 5

GROWTH OF DBA FEMALE MICE ON RATIONS CONTAINING DIFFERENT PROPORTIONS OF FAT (EXPERIMENT 2)

		Age of mice (weeks)									
Group	Dietary fat (per cent)	12	16	20	24	28	36	44	52	60	68
		Mean body weight (gm.)									
AP1	2.0	21	23	24	26	26	29	31	30	31	32
AP2	4.1	21	24	25	27	27	30	31	31	32	32
AP3	8.0	22	24	25	26	27	30	31	31	32	31
AP4	16.0	21	24	25	26	27	30	31	30	31	31
AP5	23.7	21	24	24	26	27	30	31	30	30	30

TABLE 6

FORMATION OF MAMMARY CARCINOMA IN DBA MICE FED RATIONS CONTAINING DIFFERENT PROPORTIONS OF FAT

Group	Dietary fat (per cent)	Number* of mice	Age of mice (weeks) Per cent mice with mammary tumors 50	60	70	80	90	104†	Age at appearance of tumors (weeks) Range	Mean	50 per cent age‡ (weeks)	Number of mice alive and tumor-free at 104 weeks†
AP1	2.0	54	2	15	30	45	57	74	50–104	76.3±2.4	84	5
AP2	4.1	56	5	16	36	50	75	77	40– 94	72.4±2.0	81	5
AP3	8.0	49	8	22	41	53	74	84	45–102	71.8±2.4	78	2
AP4	16.0	54	6	32	52	72	82	89	40–100	69.4±1.9	70	1
AP5	23.7	54	7	30	54	63	78	87	32– 96	68.8±2.2	70	1

* Adjusted totals, which correct for deaths of non-tumor mice during the experiment (6).

† End of experiment.

‡ Age at which 50 per cent of the mice had tumors.

alent to an increase in caloric intake, which is known to augment the rate of formation of tumors (15, 19).

The data of Forbes, Swift, and co-workers indicate that increasing the proportion of fat, in equicaloric diets, from 2 to 30 per cent causes a decreasing energy expense of utilization and a corresponding gain in body energy. For mature rats, with cage activity excluded, this difference might amount to as much as 9 per cent of the total gross energy of the diet (8, 10). However, for both young and mature rats not restricted in activity, the difference in net body energy gain between animals on 30 per cent and those on 2 per cent fat diets appears no greater than 2 to 3 per cent of the total gross energy of the diet (2, 3, 7, 9). This is equivalent to 0.2 to 0.3 Calories per day for a mouse consuming 10 Calories and allowed normal activity. These latter conditions are comparable to those employed by Boutwell, Brush, and Rusch and to those in the experiments reported in this paper.

It is our opinion that the results of Forbes, Swift, and co-workers were inadvertently misapplied by Boutwell, Brush, and Rusch (4). First, they employed the data (8, 10) concerned with resting animals (restricted activity) rather than the data dealing with animals permitted unrestricted cage activity (2, 3). Second, they did not discriminate between the gross energy *of the total diet* and the gross energy *of the supplement* (the supplement being that part of the diet supplied above the maintenance level).[2] In Figure 2, page 745, of their paper, they used the data of Forbes, Swift, and co-workers (8, 10) that refer to the energy expense of utilization of the supplement. However, Boutwell, Brush, and Rusch, in their calculations, treat the curve as if it applied to the gross energy of the total diet. Thus, they calculated a value of 1.4 Calories as the difference between the dynamic effects of the 10-Calorie rations containing 2 or 27 per cent fat, instead of the value of approximately 0.3 Calories, applicable to their experimental conditions and ours (see preceding paragraph).

This increase, approximately 0.3 Calories, in net body energy gain is definitely not large enough to produce the enhancement of tumor formation reported by us in this paper or by Boutwell, Brush, and Rusch. It would actually require an increase in net body energy, or an increase in caloric intake, of approximately 1.0–1.5 Calories per day to produce the observed effects on tumor formation.

The application of data on specific dynamic action obtained with rats to the interpretation of results on tumor formation in mice appears valid in this instance, as was indicated by Boutwell *et al.* Probably, many mammals expend less energy on utilization of high-fat diets (5), and it is unlikely that the mouse differs greatly in this respect from the rat. However, there are no data to support the view that for the mouse there is a daily net energy "sparing" of 1.4 Calories resulting from ingestion of 10 Calories of a high-fat diet as compared with one containing only 2 per cent fat. If the sparing effect were this large, one might expect the mice fed high-fat diets to weigh at least 1.5 to 2.0 gm. more[3] than those fed low-fat diets, and this does not consistently occur, as can be seen from our data and those of Boutwell, Brush, and Rusch.

More significantly, if the augmenting effect of high-fat diets on tumor formation were mediated mainly through the increased net body energy gain, one would expect that high-fat diets would enhance the formation of all types of tumors affected by the level of caloric intake. However, of all the tumors of the mouse which respond to varying the level of caloric intake, only the formation of the spontaneous mammary carcinoma and tumors of the skin induced by ultraviolet radiation or carcinogenic chemicals are modified by fat-enrichment of the diet (16), whereas the incidence of induced sarcoma, spontaneous lung adenoma, induced leukemia, and spontaneous leukemia is not affected (12, 16).

Considering it as an effective increase in available calories, the small increase in net body energy that accompanies the consumption of a fat-enriched diet may be a minor factor in the enhancement of tumor formation. However, attempts to relate the mechanism through which fat-enriched diets enhance tumor formation to the mechanism through which caloric intake exerts its influence seem premature, inasmuch as so little is known about either mechanism.

SUMMARY

The primary objective of the study was to determine the relationship between the proportion of fat in the diet and the resultant enhancement of the formation of spontaneous mammary carcinoma. One experiment utilized C3H strain mice,

[2] In long-term experiments, with growing animals, it is difficult to accurately ascribe the exact value of the dietary "maintenance" level, and consequently of the "supplement"—in fact, these change with increasing weight of the mice. According to Boutwell *et al.* (4), 8 Calories maintained mice at 25–26 gm. throughout the experiment, whereas 10 Calories maintained mice at 31–32 gm. during the major part of the experiment. Thus, for their group on the 10-Calories ration, the "supplement" may be considered to have decreased from 2 to 0 Calories during the course of the experiment.

[3] This estimate is based on data from experiments employing graded caloric restriction (4, 17).

with diets based on commercial components and four levels of dietary fat: 1.6, 5.7, 12, and 26 per cent; the second experiment utilized dba strain mice, with diets composed of semi-purified components and five levels of dietary fat: 2, 4, 8, 16, and 24 per cent. The diets were isocaloric, and the mean body weights were similar.

In both experiments, the rate of formation of mammary carcinoma, as measured both by incidence of tumors and by the average time of tumor appearance, tended to increase with increasing proportions of dietary fat. However, the effect was not arithmetically proportional to the level of fat: the enhancement of tumor formation resulting from increasing the dietary fat from 2 to 6 or 8 per cent was as great as that resulting from increasing the dietary fat from 6 or 8 per cent to 24 or 26 per cent. In contrast to the effect on tumor formation, phenomena related to the growth of the mammary carcinomas—increase in tumor size, survival time of the animal after appearance of the tumor, or the incidence of grossly visible metastases to the lungs—were not affected by the proportion of dietary fat in the range studied.

The decrease in energy expenditure of utilization of high-fat rations is too small to account entirely for the observed enhancement of tumor formation. Other suggestions are made, but it must be concluded that, at present, the mechanism through which fat-enriched diets accelerate the formation of spontaneous mammary carcinoma and carcinogen-induced skin tumors of the mouse is not known.

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The Effect of Added Dietary Tryptophane on the Occurrence of 2-Acetylaminofluorene-induced Liver and Bladder Cancer in Rats*

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Recent reports by Bielschowsky (1), Harris (6), Engel and Copeland (5), Strombeck and Ekman (10), and Morris *et al.* (7), indicate that the genetic constitution of the rat plays a more dominant role than diet in the initiation of 2-acetylaminofluorene-induced neoplasms. While liver tumors occur in a majority of the rats and the frequency is altered somewhat by the protein content of the diet, the mechanism of tumor formation differs from that of dimethylaminoazobenzene carcinogenesis, in that liver extract and riboflavin exert no protective effect against the action of this drug. 2-Acetylaminofluorene-induced mammary gland and bladder neoplasms appear to be peculiarly strain limited.

In a preliminary survey of five strains of rats, Dunning, Curtis, and Madsen (2) showed that 2-acetylaminofluorene-induced bladder tumors were confined to the Copenhagen and A×C strains and that tumors of the mammary gland were relatively infrequent, with only two observed in rats of the Fischer strain and one each in rats of the August and A×C strains. Tumors of the liver occurred in rats of all five strains but were less frequent in rats of the Copenhagen strain. A later study (4) of the occurrence of diethylstilbestrol-induced tumors in rats of three of these strains showed that mammary tumors were most frequent in the A×C rats, occurred with low frequency in Fischer rats, and failed to occur in rats of the Copenhagen strain, while the induced bladder cancers were most frequent in the Copenhagen rats, occurred with low frequency in rats of the A×C strain, and failed to occur in Fischer rats.

Rats of the Fischer strain were chosen for the present study because of their relatively high incidence of liver tumors and a low incidence of mammary tumors which might serve as comparative material for the recently reported study (3) of diethylstilbestrol-induced mammary cancer in A×C rats on similar diets. This report showed that the addition of 1.4 per cent DL-tryptophane to a synthetic diet containing 25 per cent tryptophane-free casein hydrolysate increased the number and percentage of induced cancers, and significantly reduced the average latent period below that observed for the control group on 26 per cent casein. The substitution of 4 per cent dietary tryptophane for tryptophane-free casein hydrolysate, however, produced the reverse effect—a decreased number and percentage of induced cancers and a significantly prolonged latent period.

MATERIALS AND METHODS

Pedigreed female rats of Fischer Line 344 4–5 months of age were used for these experiments. Each rat was housed in an individual cage with free access to water. The daily portion of 7 gm. of food was weighed out and presented in a food cup, to which the daily supplement of crystalline vitamins was added. Attempts were made to recover, weigh, and record all food that was spilled.

The four diets differed only in quantity and composition of the protein, as shown in Table 1. The following crystalline vitamins[1] per kilo of diet were fed as a supplement: thiamin, 4 mg.; riboflavin, 8 mg.; pyridoxine HCl, 4 mg.; niacin, 4 mg.; calcium pantothenate, 20 mg.; choline chloride, 2,000 mg.; and alpha-tocopherol, 150 mg.

Each gram of diet was equivalent to 4.7 calories, and a daily portion of 33 calories was fed.

Each rat was weighed and inspected for tumors once a week. At death, post mortem examination included description of every visible tumor, gross sectioning of lungs and mammary glands, and the inspection and weight of the liver, kidneys, adrenals, pituitary, and sex glands. Representative

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[1] Supplied through the courtesy of Dr. R. C. Pogge of Merck & Co., Rahway, N.J.

sections of each of these tissues and organs were preserved and examined microscopically.

RESULTS

The number of rats in each group, their average initial body weight, daily food consumption, and average daily and total dose of 2-acetylaminofluorene, is shown in Table 2. The twelve control rats received Friskie Dog Pellets *ad libitum*. The amount consumed was not recorded. Each group on the four synthetic diets averaged somewhat less than 7 gm. or 33 calories, which were fed to every rat daily.

The rats on diet No. 8, with the highest per cent of added tryptophane, most nearly met our experimental plan by the average consumption of 6.5 gm. of diet, or 31 calories containing 3.9 mg. of 2-acetylaminofluorene. The presence of as much as 0.06 per cent 2-acetylaminofluorene made the diet sufficiently unpalatable that the rats on all of these diets consumed less than the A×C rats (3) on similar diets without the drug. The rats on diet No. 9 with 26 per cent casein consumed an average of 5.9 gm., and those on diet No. 4 with 1.4 per cent tryptophane averaged only 5.8 gm. in daily consumption.

The post mortem weights for the rats which survived the various dietary regimens for at least 100 days are shown in Table 3. The control rats grew progressively, and the rats on diets Nos. 6 and 9 with 45 and 26 per cent, respectively, of casein, maintained their weight or gained slowly. Both groups, however, with the added tryptophane showed evidence of amino acid imbalance or caloric restriction. The weight loss was greater in the rats on the 4.3 per cent tryptophane diet.

TABLE 1

COMPOSITION OF THE DIETS

Material	Diet No. 6	Diet No. 4	Diet No. 8	Diet No. 9
	(Per cent)			
Casein	45.0	0	0	26.0
Tryptophane-free casein hydrolysate*	0	25.0	22.0	0
Salt mixture	4.0	4.0	4.0	4.0
Cellu flour	2.0	2.0	2.0	2.0
Dextrin	34.0	54.0	54.0	54.0
Crisco	15.0	15.0	15.0	15.0
Halibut liver oil	0.4	0.4	0.4	0.4
DL-tryptophane	0	1.4	4.3	0
2-Acetylaminofluorene	0.06	0.06	0.06	0.06

*Vacuum oven dried casein hydrolysate Batch Sab-A-79, tested and supplied through the courtesy of Dr. Charles F. Kade of the Sterling-Winthrop Research Institute, Rensselaer, N.Y.

They averaged 150 gm. at the start of the experiment and progressively decreased to an average of only 86 gm., while those on the 1.4 per cent tryptophane diet averaged 130 gm. at the start of the experiment and 106 gm. at death.

In all the 2-acetylaminofluorene-fed rats the livers, including the induced tumors, were relatively large and the uteri small and atrophic. Otherwise, there appeared to be no significant changes in the organs that were weighed.

TABLE 2

THE NUMBER OF RATS IN EACH GROUP, THEIR AVERAGE INITIAL BODY WEIGHT, DAILY FOOD CONSUMPTION, AND DOSE OF 2-ACETYLAMINOFLUORENE

Group	Number of rats	Body weight (gm.)	Daily ration (gm.)	Daily ration (calories)	Average dose AAF (gm.)	Average daily dose AAF (mg.)
Control	12	130			0	0
Diet No. 6	13	130	5.9	27	1.1	3.5
Diet No. 4	12	130	5.8	27	1.4	3.4
Diet No. 8	13	150	6.5	31	1.2	3.9
Diet No. 9	27	144	6.1	29	1.0	3.7

The tumor history of the rats of each group is shown in Table 4 and Figure 2 and graphically represented for each rat in Figure 1. The control group that received no 2-acetylaminofluorene had no tumors. However, the group on diet No. 9, the 26 per cent casein diet plus 0.06 per cent of 2-acetylaminofluorene, which was set up as the control for the added and deficient tryptophane diets, made the poorest record, both from the point of view of survival and tumor incidence. Eleven rats died without tumors less than 100 days from the start of the experiment. The average survival period was 283 days. Only six of the surviving sixteen developed malignant liver tumors, and only three of these had demonstrable metastases to the lungs. Seven additional rats of this group had well developed, probably benign, hepatomas. It was difficult to classify some of these tumors, and several like the one shown in Figure 3 were considered malignant—not on the basis of morphology, but by the presence of lung metastases as shown in Figure 4. Three of the rats on this diet developed squamous-cell carcinomas of the external auditory meatus, and no bladder cancers were observed.

Eleven of the twelve rats on diet No. 6 with 45 per cent casein developed malignant liver cancers, and the twelfth died, after 117 days, with benign liver tumors. Extensive lung metastases were observed in 8, or 73 per cent, of these tumor rats. Figures 3, 4, 7, and 8 are representative illustrations of the tumors induced in rats of this group. No tumors of the external auditory canal or bladder were observed.

The rats on diet No. 4 with 1.4 per cent added dietary tryptophane survived an average of 400 days or considerably longer than any other, except for the untreated controls. Eight of the eleven developed malignant hepatomas, and lung metas-

tases were demonstrable in seven of these. One of these tumors has been successfully transplanted and is now in the seventeenth generation. Figures 5 and 6 are from the tenth transplanted generation. This tumor regularly metastasizes to the lungs from the subcutaneous growths and kills the host in an average of 60 days. Hepatoma No. 6, shown in Figure 7, has also been successfully transplanted subcutaneously. It is now in the 21st transplanted generation, and, although it shows more mitosis and less cellular differentiation, no metastases have been observed from the subcutaneous growths. In addition two rats on diet No. 4 developed squamous carcinoma of the external auditory meatus, three had what were considered benign hepatomas, and all eleven developed carcinoma of the bladder. Figure 10 shows a section of the first transplanted generation of one of these tumors. The majority of the tumors were papillary carcinomas, as shown in Figure 9, but areas of squamous carcinoma were not uncommon.

The rats on diet No. 8 with 4.3 per cent of

TABLE 3

THE NUMBER OF RATS WHICH SURVIVED FOR AT LEAST 100 DAYS, THEIR AVERAGE POST MORTEM BODY WEIGHTS IN GRAMS, AND THE PERCENTAGE WEIGHTS OF SOME OF THE ORGANS

Group	Number of rats	Average body wt.	Organ Weights in Per Cent of Body Weight					
			Liver	Kidney	Adrenal	Pituitary	Ovary	Uterus
Control	12	174	4.8	1.0	.04	.01	.06	.56
Diet No. 6	12	138	19.3	1.3	.03	.01	.04	.23
Diet No. 4	11	106	16.4	1.3	.04	.01	.04	.21
Diet No. 8	12	86	15.7	1.5	.05	.01	.03	.18
Diet No. 9	16	136	11.7	1.2	.04	.01	.04	.28

TABLE 4

THE AVERAGE SURVIVAL IN DAYS, THE NUMBER AND PERCENTAGE OF RATS IN EACH GROUP WITH LIVER, BLADDER, AND EAR CANCERS, AND THE NUMBER AND PERCENTAGE WITH LUNG METASTASES OF THE LIVER CANCERS

Group	Number of rats	Average survival	Liver ca.		Lung met.		Bladder ca.		Ear ca.	
			(No.)	(per cent)	(No.)	(per cent)	(No.)	(per cent)	(No.)	(per cent)
Control	12	406	0	0	0	0	0	0	0	0
Diet No. 6	12	303	11	92	8	73	0	0	0	0
Diet No. 4	11	400	8	73	7	88	11	100	2	18
Diet No. 8	12	320	9	75	7	78	11	92	0	0
Diet No. 9	16	283	6	37	3	50	0	0	3	19

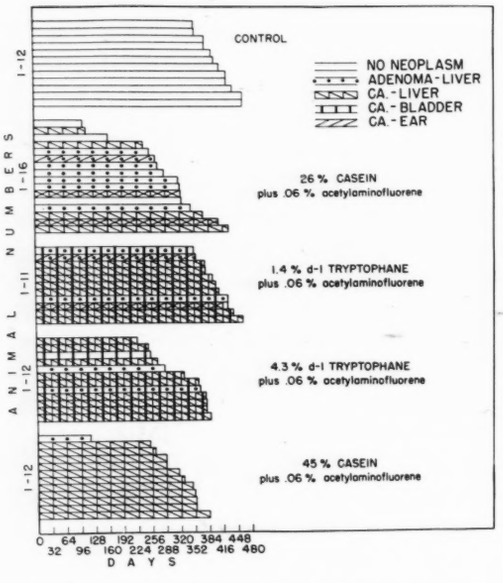


FIG. 1.—Survival period and tumor history of rats on each of the several dietary regimens. (Each rat is represented by a bar, the length of which indicates the period of survival; the pattern as shown in the key indicates the character of the neoplasm identified in the post mortem study.)

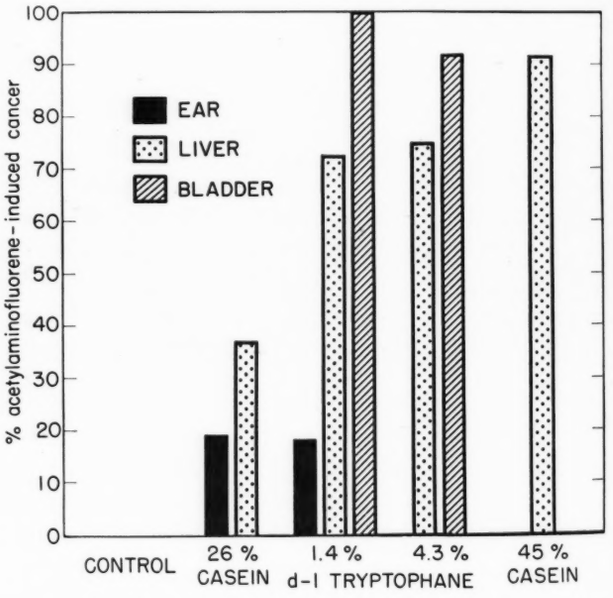
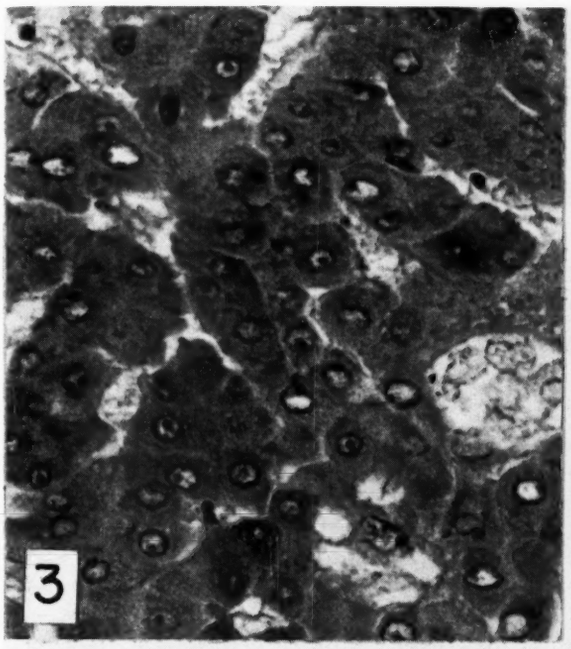
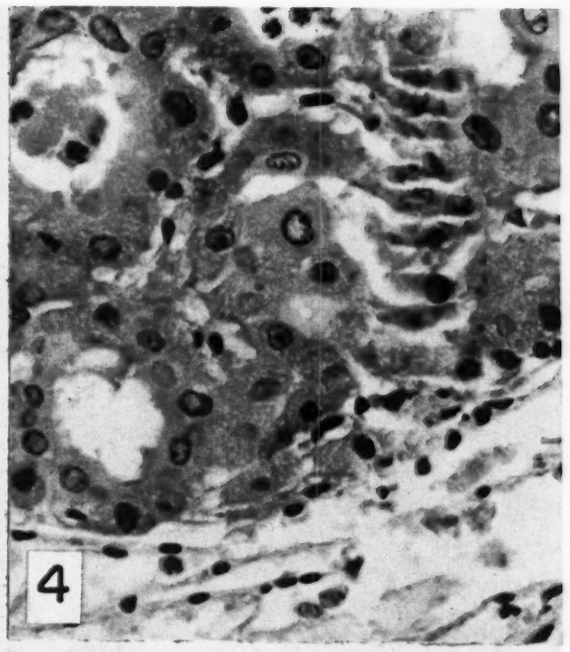
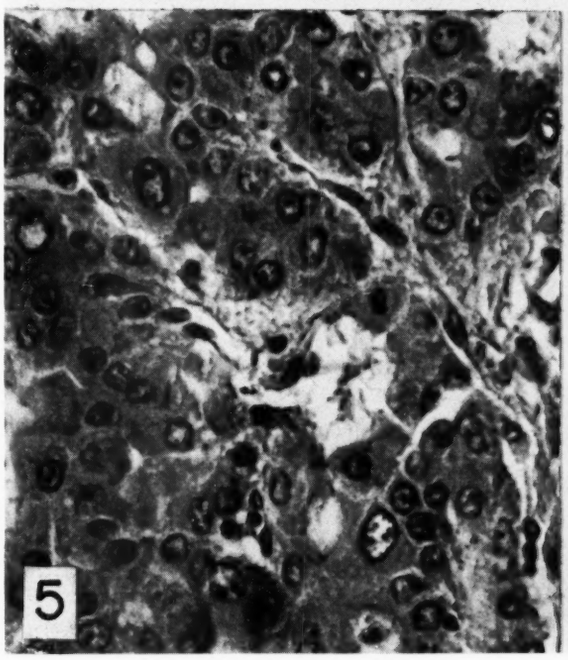


FIG. 2.—The per cent of neoplasms of each category that were identified in the rats on the several dietary regimens.







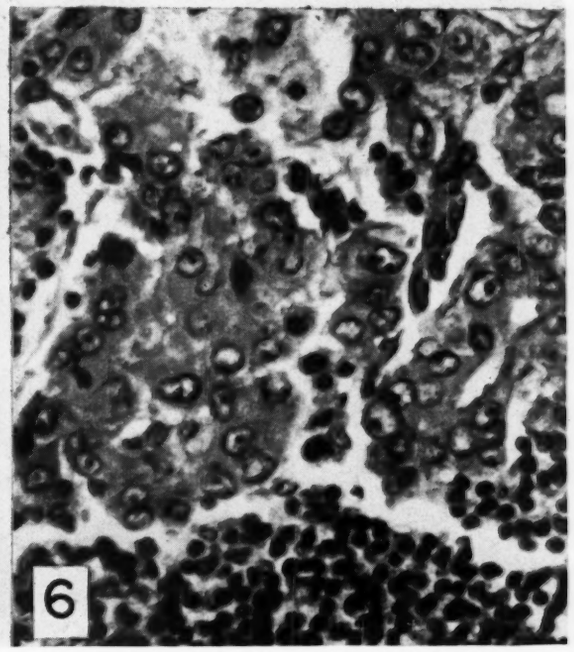


FIG. 3.—Hepatoma No. 9 from rat maintained 353 days on 45 per cent casein diet. × 280.

FIG. 4.—Lung metastasis of hepatoma No. 9. × 280.

FIG. 5.—Tenth transplanted generation of hepatoma No. 18 induced in rat maintained 460 days on 1.4 per cent tryptophane diet. × 280.

FIG. 6.—Lymph node metastasis from hepatoma No. 18. × 280.

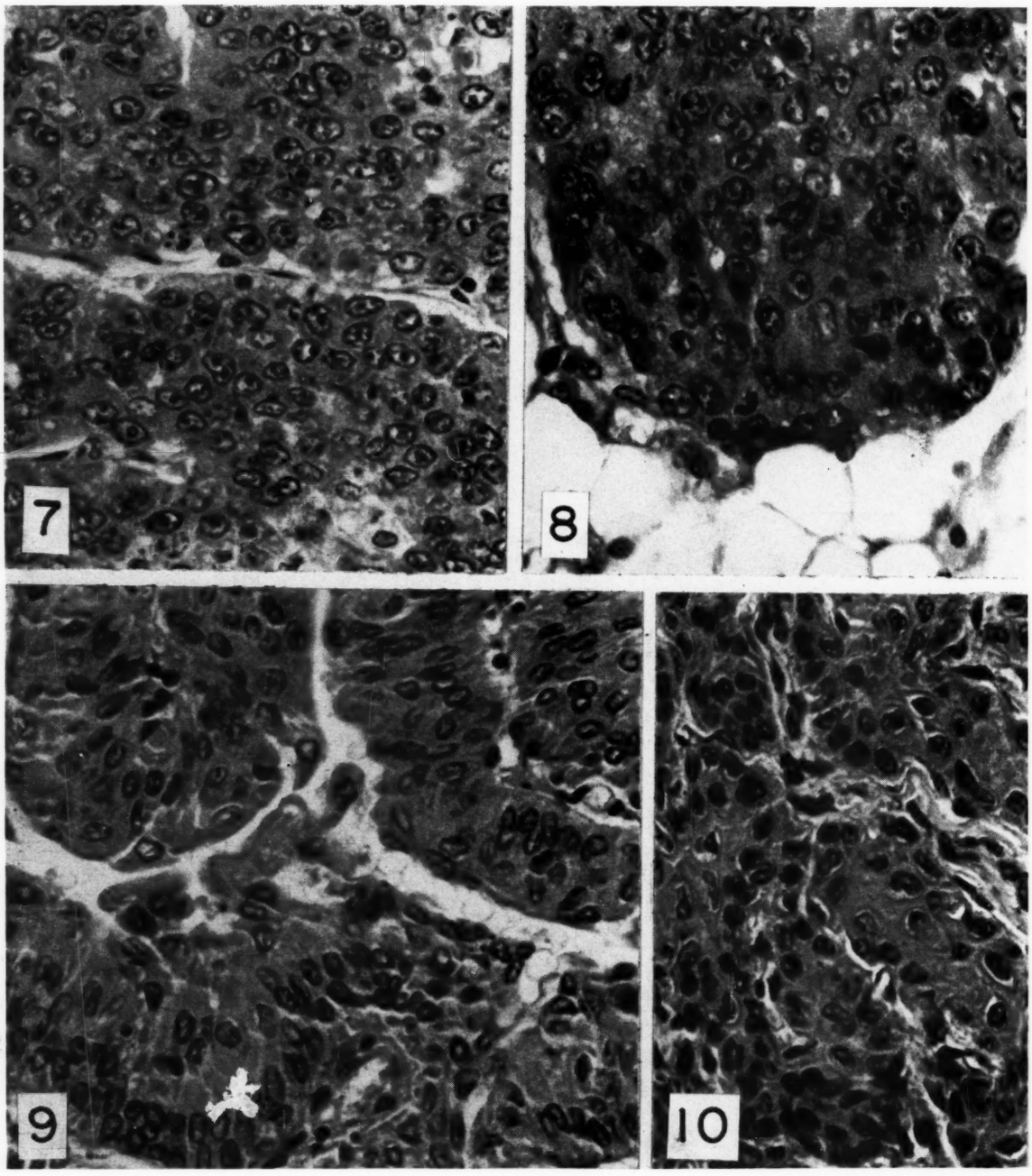


FIG. 7.—Hepatoma No. 6 from rat maintained 326 days on 45 per cent casein diet. × 280.

FIG. 8.—Omental metastasis of hepatoma No. 6. × 280.

FIG. 9.—Papillary carcinoma No. 12 of bladder from rat maintained 376 days on 4.3 per cent tryptophane diet.

FIG. 10.—First transplanted generation of bladder carcinoma No. 19 induced in rat maintained 460 days on 1.4 per cent tryptophane diet. × 280.

added dietary tryptophane developed liver cancer in about the same proportion as rats of the former group. That is, nine of the twelve had malignant hepatomas, with demonstrable lung metastases in seven, and two had probably benign hepatomas. Eleven of these rats also developed bladder cancer and in a relatively shorter interval than those of the former group. The average survival period of these rats was 320 days, compared with 400 days for those on diet No. 4 with 1.4 per cent added tryptophane.

The increased proportion of dietary tryptophane, or the imbalance caused thereby, may be a real factor in the initiation of these bladder cancers. No spontaneous bladder cancers have been observed in rats of this inbred line, and no bladder cancers were initiated by the diethylstilbestrol technic that successfully initiated bladder cancer in rats of the Copenhagen strain. No bladder cancers have been observed in previous or subsequent series of rats of this line, on diets in which the protein source has been casein or whole wheat flour and whole milk powder (6). Furthermore, the low body weights of these rats should have exerted a protective effect, if the theory of Tannenbaum and Silverstone (8, 11) is tenable. Their caloric intake was approximately equal to that of the other groups of rats, but the absorption or utilization must have been disturbed. The experience of Strombeck (9) with azotoluene-induced hyperplasia of the bladder mucosa in rats in which a portion of bladder mucosa had been transplanted to the liver would suggest that the changes observed in the bladder result from direct contact with the chemical in the urine. In Strombeck's experiment, sixteen rats with established grafts of bladder mucosa in the liver showed only normal mucosa in the transplant and papillomatous or hyperkeratotic changes in the mucosa of the bladder *in situ*. In the present experiment, combined elimination products of 2-acetylaminofluorene and tryptophane may be a real factor in the initiation of these bladder cancers, and diet appears to exert a more predominant role than hereditary constitution.

SUMMARY

1. Sixty-five female rats of the Fischer Line 344 were placed on isocaloric synthetic diets containing 0.06 per cent 2-acetylaminofluorene.

2. The rats were divided into four groups receiving, respectively, 45 per cent casein, 26 per cent casein, 25 per cent tryptophane-free casein hydrolysate plus 1.4 per cent DL-tryptophane, and 22 per cent tryptophane-free casein hydrolysate, plus 4.3 per cent DL-tryptophane.

3. Fifty-one rats survived for at least 100 days on the various dietary regimens and consumed a daily average of nearly 4 mg. of 2-acetylaminofluorene.

4. The rats on the 45 and 26 per cent casein diets maintained their body weight, while those on the 1.4 per cent and 4.3 per cent tryptophane diets lost, respectively, 20 and 40 per cent.

5. Benign and malignant hepatomas were observed in the majority of rats in each group. The per cent of malignant hepatomas varied from 37 in the rats on the 26 per cent casein diet to 92 in the rats on the 45 per cent casein diet and was, respectively, 73 and 75 per cent in the rats on the 1.4 and 4.3 per cent tryptophane diets.

6. Squamous carcinoma of the external auditory meatus occurred in 20 per cent of the rats on the 26 per cent casein diet and 1.4 per cent tryptophane diet.

7. Bladder cancer occurred in 100 per cent of the rats on the 1.4 per cent tryptophane diet and in 92 per cent of the rats on the 4.3 per cent tryptophane diet. No bladder cancers were observed in the rats on the 45 per cent or 26 per cent casein diets.

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Announcements and Comments

OAK RIDGE SUMMER SYMPOSIUM

The second annual Oak Ridge Summer Symposium, devoted this year to quantum and inorganic chemistry, will be held at Oak Ridge, Tennessee, from August 21 to August 31 under the joint sponsorship of the Oak Ridge National Laboratory and the Oak Ridge Institute of Nuclear Studies. The first symposium last year was in modern physics.

The program will include several lectures, each by distinguished chemists. The principal speakers and the fields of their lectures are as follows:

Dr. Peter Debye, Todd Professor of Chemistry at Cornell University and a Nobel Laureate in chemistry: "New Developments in Polymers and Colloidal Solutions."

Dr. Henry Eyring, Dean of the Graduate School of the University of Utah: "Reaction Kinetics."

Dr. Herbert S. Harned, Professor of Chemistry at Yale University: "The Present Status of Our Quantitative Knowledge of Electrolytic Solutions."

Dr. Linus C. Pauling, Professor of Chemistry and Chairman of the Division of Chemistry and Chemical Engineering, California Institute of Technology: "The Electronic Structure of Molecules and Crystals."

Dr. George Scatchard, Professor of Physical Chemistry, Massachusetts Institute of Technology: "The Physical Chemistry of Solutions."

The symposium will be free of security restrictions and will be held in an air-conditioned building in downtown Oak Ridge. There will be no admission for attendance; however, those attending will arrange for their own traveling and living expenses. Afternoons on Friday, Saturday, and Sunday will be free for informal discussions or recreational activities.

A committee comprised of Drs. G. E. Boyd, M. A. Bredig, K. A. Kraus, and H. A. Levy, all of the Oak Ridge National Laboratory, and Dr. S. C. Lind, consultant to the Carbide and Carbon Chemicals Division in Oak Ridge, has arranged the program.

Additional information on the symposium, housing, and restaurant facilities in and around Oak Ridge, and other material may be obtained from the University Relations Division, Oak Ridge Institute of Nuclear Studies, P.O. Box 117, Oak Ridge, Tennessee.

COMMENTS TO THE EDITOR

Drs. Morris, MacDonald, and Mann, in their paper on "Intra-ocular Transplantation of Heterologous Tissues" (Cancer Research, **10**: 36–48, 1950) describe survival of transplants of human cancers and normal human tissue inoculated into the lens of guinea pigs. The transplantation was "accomplished by directing the trocar beneath the capsule of the lens."

I question if a trocar (? gauge) "directed beneath the capsule of the lens" will easily penetrate this structure. We have attempted to perform this procedure on a guinea pig eye *in situ*, on a freshly removed guinea pig eye and on a freshly removed guinea pig lens, using a No. 17 and a No. 20 trocar. We were only successful when the lens capsule had been cut with a knife.

Pseudo-epitheliomatous proliferation of lens epithelium following trauma to the lens or infection of anterior chamber contents is not uncommonly seen. The histological appearance is similar to that seen in Figures 2, *c* and *d*, and Figures 3, *b* and *c*, in Dr. Morris' paper. It is doubtful if the lung tissue shown in Figure 5, *b* is located in the lens substance or if it is surrounded by the lens capaule. I question, therefore, if the reported ten successful intralenticular transplants represent survival of inoculated tissue. Histological demonstration of transplants located in the lens immediately after inoculation would prove Dr. Morris' point.

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ERRATUM

In the article entitled "Histological Changes Produced by a Single Large Injection of Radioactive Phosphorus (P^{32}) in Albino Rats and in C3H Mice, by Grad and Stevens, in May (Cancer Research, **10**: 292, 1950): line 26, which reads "At 72 hours, the thickness of the mucosa was definitely greater" should be corrected to read "At 72 hours, the thickness of the mucosa was definitely reduced (Fig. 7), but the proportion of mitoses to pyknoses was definitely greater."